EFFECTS OF HERBICIDE MIXTURES ON MICROBIAL COMMUNITIES FROM A PRAIRIE WETLAND ECOSYSTEM

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ABSTRACT

The Prairie Pothole wetlands of Saskatchewan and Manitoba serve an important ecological role in providing wildlife habitat, water storage, and water filtration and display a wide range of water quality parameters such as salinity and nutrients. These wetlands are regularly interspersed among agricultural operations where multiple pesticides are commonly used. This dissertation investigated the effects of glyphosate, an auxin-type herbicide mixture (2,4-D, MCPA, clopyralid, dicamba, dichlorprop, mecoprop), and a mixture of eight herbicides (including auxin-type herbicides, bromoxynil, and glyphosate) on pelagic and biofilm microbial communities. Three different experimental approaches were used including mesocosms, enclosures, and curtained whole wetlands. Effect assessment indices included: primary productivity, chlorophyll *a* content, bacterial productivity and numbers, protein and carbohydrate concentrations, bacterial carbon source utilization patterns, and algal pigment profiles.

In the mesocosm experiment, effects of glyphosate as well as two herbicide mixtures (as noted above) were investigated. The glyphosate concentration utilized was 1000 times the environmentally relevant concentration (ERC). One herbicide mixture consisted of six auxin-type herbicides (listed above), each at 1000 times ERC. For the second mixture (eight herbicides: listed above), a dose-response approach was used with the ERCs of each herbicide as the base concentration. Results indicated that the eight herbicides mixture, even at low concentrations, produced effects on microbial communities. Glyphosate treatment suppressed algal productivity in both pelagic and biofilm communities. Auxin-type herbicide mixture, in general, had stimulatory effect on algae. This study indicated that glyphosate is more toxic to pelagic and biofilm wetland algal communities than the auxin-type herbicide mixture.

To further investigate effects of the eight herbicides mixture (same as above) at maximum-exposure concentrations on microbial communities in ponds varying in salinity and nutrients,

four wetlands (1 freshwater and 3 saline) were selected. Six enclosures (3 controls and 3 treatments) were installed in each pond. Results demonstrated that the herbicide mixture had a stimulatory effect on primary productivity in the nutrient-sufficient freshwater pond while no stimulatory effect was observed in the nutrient-deficient saline ponds.

For the curtained wetland experiment, effects of eight herbicides mixture (same as previous) on microbial communities were investigated in an ephemeral and a semi-permanent wetland. Herbicide treatment at maximum-exposure concentration stimulated primary productivity in the ephemeral wetland likely due to the hormonal effect of auxin-type herbicides present in the mixture. In contrast, suppression of primary productivity (herbicidal effects) during the first week post-treatment was noted in the semi-permanent wetland, possibly a result of a concentration addition effect of the auxin-type herbicides. Biofilm bacterial carbon source utilization patterns and pigment profiles suggested a change in the community structure in both wetlands. This dissertation demonstrated the effects of herbicide mixtures on microbial communities using three different experimental approaches as well as in different types of wetlands.

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Dedicated to

my beloved parents

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LIST OF ABBREVIATIONS

ABA: Abscisic acid

ACC: 1-aminocyclopropane-1-carboxylic acid

AMPA: Aminomethyl phosponic acid

AUX: Auxin-type herbicide treatment

BP: Bacterial productivity

CA: Concentration addition

Chl *a*: Chlorophyll *a*

DAPI: 4,6-diamidino-2-phenylindole

DO: Dissolved oxygen

DOC: Dissolved organic carbon

EC_{50}: Concentration at which effects occur in 50 % of the test organisms

EPA: Environmental Protection Agency

EPSP: Enolpyruvyl shikimate phosphate

ERC: Environmentally relevant concentration

ETS: Electron transport system

GLY: Glyphosate treatment

IA: Independent action

LD_{50}: Dose at which mortality occurs in 50 % of the number of test organisms

LOAEL: Lowest observed adverse effects level

MOA: Mode of action

NOAEC: No observed adverse effects concentration

NOAEL: No observed adverse effects level

PP: Primary productivity

PPR: Prairie pothole region

PS I: Photosystem I

PS II: Photosystem II

SDNWA: St. Denis National Wildlife Area

TCA: Trichloroacetic acid

TDI: Tolerable daily intake

1. INTRODUCTION

Herbicides occur in aquatic ecosystem due to spray drift, aerial deposition, surface run-off, or groundwater flow. These herbicides vary in number and concentration in aquatic ecosystems depending on their proximity to croplands, amounts of herbicides used and also the conditions under which these herbicides were applied to croplands. Across the Canadian prairies, where multiple herbicides are used, it is logical to expect multiple herbicide residues in surrounding aquatic systems. Herbicide mixtures are often detected in these prairie water bodies (Cessna and Elliott, 2004; Donald and Syrgiannis, 1995; Donald et al., 1999, 2001, 2007; Grover et al., 1997).

Most studies on herbicide effects are classical toxicological studies using established species in controlled environments. These studies lead to standard EC_{50} values (concentration at which effects occur in 50 % of the number of test organisms), NOAEL values (No Observed Adverse Effects Level), LOAEL values (Lowest Observed Adverse Effects Level) or LD_{50} values (dose of a chemical administered (mg of chemical per kg of body weight) that kills half (50 %) of the animals tested). These single species laboratory studies evaluate direct toxic effects, but cannot extrapolate secondary effects or the chain of events at the community level. Although conditions in laboratory studies can be controlled (less complexity and variability), thereby limiting the range of effects, these types of studies do not reflect environmental effects (USEPA, 1998a), e.g., lack of interspecies or inter-trophic interactions and lack of buffering or restoring capacity by the components of the ecosystem in single species laboratory tests. Single-species laboratory studies provide valuable information on herbicide toxicity; however, they fail to capture synergistic or

antagonistic effects dependent on the surrounding ecosystem (both biotic and abiotic) and effects at the community level rather than species level.

Prairie wetlands are important aquatic ecosystems consisting of primary producers, bacteria, benthic invertebrates, emergent insects and higher trophic level mammals and migrating waterfowl and support 50 to 80 % of the North American waterfowl population and approximately half of other migratory birds each year (Mitsch and Gosselink, 2000). Microbial communities consisting of phytoplankton and bacteria are vital members of the wetland food web and any changes in productivity at these lower trophic levels may affect the food supply to higher trophic levels (DeLorenzo et al., 2001; Waiser and Robarts, 2004). The toxicity of herbicides to these important trophic levels has been generally overlooked. There is a large disconnect between the wetlands research and risk assessment because of the lack of wetland toxicity data and its interpretation, leading to uncertainties in protecting wetlands (Lemly et al., 1999). Consequently, there is need for more ecologically relevant wetland effects studies to reduce the uncertainty in assessing the risks of herbicides to these ecologically important aquatic systems.

The current research study investigated the effects of herbicides (2,4-D [2,4-dichlorophenoxyacetic acid], MCPA [2-methyl-4-chlorophenoxyacetic acid], dicamba [3,6-dichloro-2-methoxybenzoic acid], clopyralid [3,6-dichloropyridine-2-carboxylic acid], dichlorprop [2-(2,4-dichlorophenoxy)propanoic acid], mecoprop [2-(4-chloro-2-methylphenoxy)propanoic acid], bromoxynil [3,5-dibromo-4-hydroxybenzonitrile], and glyphosate [2-(phosphonomethylamino)acetic acid]) on sensitive endpoints in wetland microbial communities. These herbicides are among the most commonly detected in prairie wetlands

(Donald et al., 2007) and amongst the most frequently used on the prairies (Waiser and Holm, 2005). The following hypotheses were put forth:

1. The presence of herbicide mixtures with different modes of action would decrease the primary and bacterial productivity in both pelagic and biofilm communities in the wetland ecosystem due to the herbicidal activity of the herbicides.
2. Effects of herbicide mixtures would be dependent on the concentrations of each herbicide in the mixture and the auxin-type herbicides would affect the microbial communities collectively because of their similar modes of action.
3. The wetland water characteristics such as nutrient status and salinity would interfere with the overall effects of herbicide mixtures.

1.1 Research Questions and Technical Objectives

The research questions included:

(i) How does a mixture of herbicides, commonly found in prairie wetlands, affect the microbial communities?

(ii) Do the auxin-type herbicides, with similar modes of action, follow the concept of concentration addition in exhibiting their toxicity on microbial communities?

(iii) How do nutrients and salinity of prairie wetlands influence the toxicity of herbicide mixtures?

The objectives of this research were to:

(i) Investigate effects of a mixture of eight herbicides (2,4-D, MCPA, clopyralid, dicamba, mecoprop, dichlorprop, bromoxynil and glyphosate) on wetland microbial communities at 1, 10, 500 and 1000 times the environmentally relevant concentration (ERC) for each of the eight herbicides using a outdoor mecososm approach.

(ii) Investigate effects of a mixture of six auxin-type herbicides (2,4-D, MCPA, clopyralid, dicamba, mecoprop, and dichlorprop) on wetland microbial communities at 1000 times ERC for each of the six herbicides and the effects of glyphosate on wetland microbial communities at 1000 times ERC using a outdoor mesocosm approach.

(iii) Investigate effects of a mixture of eight herbicides on wetland microbial communities at concentrations based on a maximum-exposure scenario using enclosures, in four ponds varying in salinity.

(iv) Investigate effects of a mixture of eight herbicides on wetland microbial communities at concentrations based on maximum-exposure scenario using a whole-wetland approach in two hydrologically different wetlands.

The herbicides investigated here are those commonly used across prairie farms and also those commonly detected in prairie wetlands (Donald et al., 2007; Waiser and Holm, 2005). Knowledge of microbial community responses to these herbicides will help in understanding the risks presented to the food chains within these ecologically significant and biologically diverse wetland ecosystems. It will also help in making informed decisions to mitigate adverse effects and protect aquatic ecosystems. The importance of wetlands and the biodiversity within them is stressed well enough in the literature and there is a need to protect and conserve these wetlands. In order to maintain and sustain the integrity of these important ecosystems, understanding herbicide mixture effects is imperative.

The research findings from this dissertation are presented as manuscripts: In Sura et al. (2012), (published in Journal of Environmental Quality) (Chapter 3), results from effects of glyphosate, the mixture of six auxin-type herbicides, and a mixture of eight herbicides

(objectives i and ii) using an outdoor mesocosm approach were presented and discussed in detail. In Sura et al. (manuscript) (chapter 4), results from the enclosure study in four ponds with varying salinity (objective iii) were presented and discussed. In Sura et al. (2012, accepted for publication in Science of the Total Environment) (chapter 5), results from the whole-wetland study (objective iv) were presented and discussed. Chapters 3 to 5 contain an appropriate literature review relevant to each manuscript. In addition, a broader literature review on various subjects relevant to this dissertation is provided in chapter 2. Chapter 6 contains a general synopsis and conclusions of the findings of this research. And the last chapter (Chapter 7) contains the bibliography for this dissertation.

2. LITERATURE REVIEW

2.1 Wetlands

Wetlands encompass many different habitats including ponds, marshes, swamps and peatlands. Many wetlands have formed in shallow depressions or on floodplains associated with estuaries, rivers, lakes, and streams. Some have become established in poorly drained depressions, many of which are completely surrounded by upland. These types of wetlands are referred to as "isolated" wetlands (Dammon and French, 1987; Tiner, 1996). There is no universally accepted definition of wetlands (Catallo, 1993). They are areas where land and water meet and are wet for an ecologically-significant part of the year. Cowardin et al. (1979) defined wetlands as lands where ecosystem development including soil development is influenced by a dominant factor - water saturation. The Environmental Protection Agency (EPA) under the Clean Water Act (USEPA, 1970) defines wetlands as areas that are flooded or saturated with water, either surface or ground, at regular intervals and for sufficient duration of time to support vegetation adapted to the soil conditions. Wetlands function as ecotones, transitions between different habitats, and have characteristics of both aquatic and terrestrial ecosystems. They are areas of transition zones between uplands and open water. The hydrogeological conditions and the physical, chemical and microbiological properties of the bottom sediments and plant communities are the defining characteristics of wetlands (Catallo, 1993). Wetlands may be temporally flooded each day as with tidal marshes, or be filled seasonally with water from melting snow, or filled permanently with water recharge from groundwater. These transition zones provide habitat for living organisms from both the land and water making them highly

productive environments. The net primary productivity of wetlands greatly exceeds those of grasslands, cultivated lands and most forests, making them the most productive ecosystems in the world (Richardson, 1995).

Wetlands are found globally except in Antarctica (Ornes, 2008). More than 6 % of the earth's surface (~ 8.6 million square kilometres) is covered with wetlands (Mitsch and Gosselink, 2000). Matthews and Fung (1987) estimated the extent of wetlands in various climatic zones around the world. They concluded that about 50 % of wetlands are in tropical and subtropical regions while the remaining are primarily polar / boreal peatlands in arctic and subarctic regions. Wetlands in the temperate zone account for 1 to 2 % of the total. Canada possesses 25 % of the world's wetlands, and 18 % of its landmass (>127 million hectares) is covered by these water bodies (Hebert, 2000). Many original wetland areas, however, have been converted or drained. Although wetlands are generally smaller than lakes, they occupy huge regions. The prairie pothole region (PPR) across south central Canada and the north central United States, for example, contains more than four million wetlands and ponds. Over the past several decades, the total wetland area in North America has declined substantially, mostly due to human activities (Dahl, 2000). About 70 % of wetlands have already been lost to human settlement, and industrial and agricultural activities (DUC, 2006). Although there may be no way to precisely estimate total wetland area lost as a result of human interferences, it can be roughly estimated by taking into account various human settlements that took over former wetlands. For example, Mexico City is the site of a wetland that was lost to human settlements 400 years ago. Similarly, most of the major airports in United States are situated on former wetlands (Mitsch and Gosselink, 1993).

2.1.1 Prairie Pothole Region (PPR)

The PPR covers nearly 800 000 sq. km of five U.S. states (Montana, North Dakota, South Dakota, Minnesota and Iowa) and three Canadian provinces (Alberta, Saskatchewan, Manitoba)

(Figure 2.1). Approximately 15 to 25 % of the prairie region is wetland and 11 % of Canada's wetlands are present in Saskatchewan (Mitsch and Gosselink, 2000). Wetlands of this region are formed as a result of glacial retreat during the Pleistocene Epoch (1,808,000 to 11,550 years BP (Before Present)). The last glacial retreat from this region was approximately 12,000 years ago when water from the melting ice formed large glacial lakes. Large pieces of ice buried in the newly deposited soil subsequently melted forming wetlands (Euliss et al., 1999). This region is underlain by glacial till of Battleford and Floral Formations (Hayashi, 1996; Miller, 1983).

The PPR has a continental climate with low and variable precipitation. Air moves into the northern prairie region from the Pacific Ocean and the Arctic and much of the moisture in the air is removed by the mountains to the west such that relatively dry air reaches the prairies. The mean annual precipitation is 352 mm, 27 % of which falls as snow. Most summer rainfall is of short duration but high intensity, typical of convective storms in a semi-arid climate (Su et al., 2000; van der Valk, 1989). Prairies have a negative water balance with respect to the atmosphere. For example, precipitation minus evaporation ranges from -10 cm in Iowa to -60 cm in southwestern Saskatchewan (van der Valk, 1989).

Prairie wetlands are fed mainly by snow, with 25 % as snowfall directly on wetlands and over 50 % as snowmelt and run-off. Other sources include precipitation and run-off from the surrounding agriculture fields. Water levels fluctuate seasonally, typically peaking in spring and declining throughout summer (Euliss and Mushet, 1996; Euliss et al., 1999). In addition to seasonal fluctuations, water levels in wetlands vary greatly from year to year. Drought for prolonged periods may dry up small wetlands. Intermittent droughts prove to be stimulating for seed growth rather than destructive such that wetland sediments contain seed banks. When water levels decrease and bottom sediments are exposed, terrestrial and drought tolerant plant species

germinate (Harris and Marshall, 1963). When such wetlands reflood, the terrestrial species are completely replaced by emergent and submerged plant species (Euliss et al., 2004; Harris and Marshall, 1963). Wetlands may be classified based on various characteristics such as hydrology, specific conductance, etc. Seven major classes of wetlands are recognized based on the ecological differentiation, hydrological and geological conditions and vegetation (Stewart and Harold, 1971): Class I (ephemeral ponds), class II (temporary ponds), class III (seasonal ponds and lakes), class IV (semi-permanent ponds and lakes), class V (permanent ponds and lakes), class VI (alkali ponds and lakes), class VII (fen ponds (alkali bogs)). Wetlands may be classified into different types based on the specific conductance of water: fresh (< 500 μS/cm), slightly brackish (500 – 2000 μS/cm), moderately brackish (2000 – 5000 μS/cm), brackish (5000 – 15000 μS/cm), subsaline (15000 – 45000 μS/cm), and saline (> 45000 μS/cm). Change in water levels is the main factor contributing to different salinities. High salinity is usually associated with loss of water by evapo-transpiration or greater inflow of water from the groundwater table (Stewart and Harold, 1971).

Based on the time of water flooding, there are temporary, seasonal (ephemeral), and permanent wetlands. Temporary wetlands are flooded for a short time in the spring or after heavy precipitation. Seasonal wetlands normally are flooded with water in spring and early summer, while permanent wetlands are flooded year-round. Most temporary wetlands dry out by end of summer while the permanent ones may dry out during extreme droughts (Huel, 2000; Robarts et al., 1995). The unique geological history, hydrology and climate of the prairie region have a great influence on the water chemistry, flooding period, and ultimately the biotic communities that inhabit these wetlands (Euliss et al., 1999).

The semiarid glaciated plains of the North American continent have unique hydrological and hydrogeological characteristics due to the combination of the semi-arid, cold climate and the glacial deposits that underlay the area. The glacial deposits are a rich source of mineral nutrients and the closed nature of the basins means that the nutrients are trapped and recycled in the wetlands instead of being flushed out by surface runoff. Prairie wetlands located in lower parts of the landscape in a given area are generally more saline than those located in the higher parts (LaBaugh, 1989; Sloan, 1972). The glacial deposits are the source of most of the sulfate salts that occur in soils and in and around wetlands and lakes of the northern prairie region. At the St. Denis National Wildlife Area, Saskatchewan, Canada, for example, electrical conductivities of pond water range from less than 400 μS/cm for shallow marshes to over 24000 μS/cm for terminal wetlands, with sulfate as the dominant anion for the more saline ponds (Driver and Peden, 1977). The difference in salinity is reflected in the composition of plant communities (Stewart and Kantrud, 1972).

Wetlands in the PPR serve an important role in providing wildlife habitat, and water storage and filtration. Prairie wetland food-webs consist of primary producers (free-living and attached algae, submerged and emergent plants), bacteria, benthic invertebrates, emergent insects and higher trophic level mammals and migrating waterfowl. These aquatic ecosystems are key ecological features of the prairie region, supporting 50 to 80 % of the North American waterfowl population in any given year (Batt et al., 1989). Half of the migratory birds on the continent utilize the wetlands in PPR.

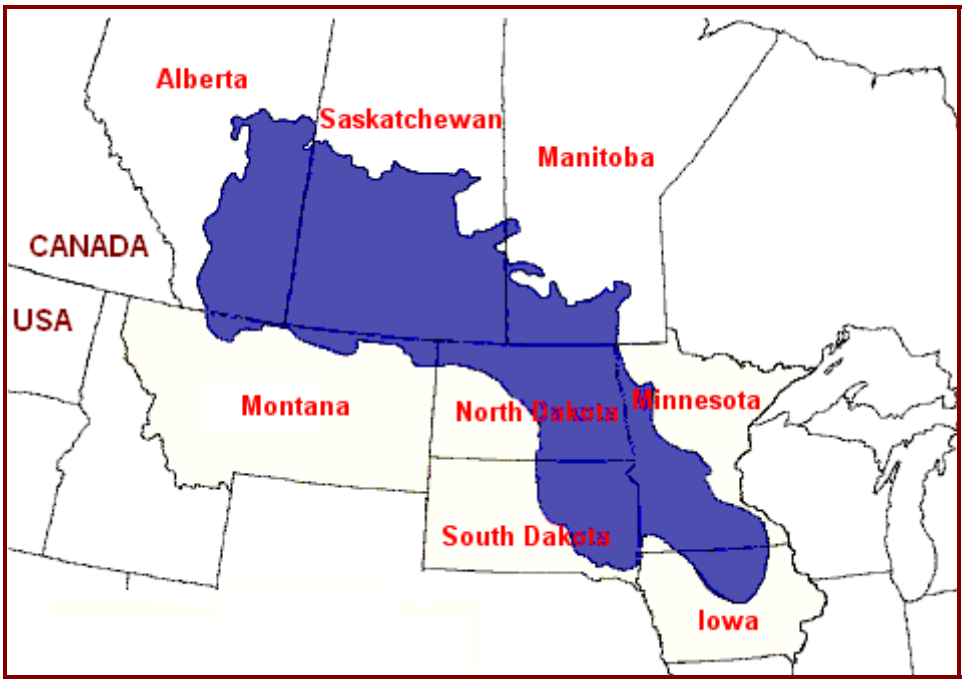


Figure 2.1 Area covered by Prairie Pothole Region (PPR) in Canada and U.S.A. Modified figure from U.S. Fish and Wildlife Service, http://www.fws.gov/kulmwetlands/pothole.html (accessed 2 November 2011).

2.1.1.1 St. Denis National Wildlife Area (SDNWA)

The St. Denis National Wildlife Area (SDNWA), part of PPR, is located in the mixed-grass prairie eco-district, 40 km east of Saskatoon, Saskatchewan, Canada (52^{o} 02' N 106^{o} 05' W). It has over 200 temporary and permanent wetlands ranging from freshwater to saline, covering an area of 361 ha (EC, 1983). Major habitat types within SDNWA include cultivated land (41 %), wetlands (21 %), tame grasslands (19 %), native grasslands (16 %), and woodlands (3 %). Most of the wetlands of this region provide habitat for many species of waterfowl, songbirds, hawks, owls, and various mammals (EC, 1983).

2.1.2 Pesticides in Prairie Pothole Region

The PPR is an example of wetland-agriculture landscape association in North American continent. Wetlands in this region are embedded among intensive agricultural operations where herbicides, fungicides, and insecticides are commonly used (Donald et al., 1999; Waiser and Robarts, 1997). Crop-production is a major industry in the Canadian prairies and most cultivated

land in this region is treated with pesticides to maximize yield. These pesticides eventually reach the surrounding wetlands via processes like spray drift, aerial deposition, surface runoff, or groundwater flow (Grover et al., 1988; Waite et al., 1992) and pesticides are often detected in prairie wetlands (Donald and Syrgiannis, 1995; Donald et al., 1999, 2001, 2007; Waite et al., 2004). Among pesticides, herbicides continue to be the most frequently used in Canada. Among total pesticide sales in 2009, for example, 76 % were herbicides [CropLife Canada, http://www.croplife.ca/web/english/plant_science_industry/ (accessed 3 May 2010)].

Grover et al. (1997) monitored seven commonly used herbicides in 21 Saskatchewan farm dugouts. Water samples were collected in the spring following snowmelt, in summer (mid-July), when herbicide application is normally completed, and in the fall before ice formation. At least one herbicide was detected in each sample collected. Diclofop (methyl-2-[4-(2,4-dichlorophenoxy)phenoxy]propionate), 2,4-D (2,4-dichlorophenoxyacetic acid), bromoxynil (3,5-dibromo-4-hydroxybenzonitrile), MCPA (2-methyl-4-chlorophenoxyacetic acid), triallate ((2,3,3-trichloro-2-propenyl)bis(1-methylethyl)carbamothioate), dicamba (3,6-dichloro-2-methoxybenzoic acid) and trifluralin (2,6-dinitro-*N*,*N*-dipropyl-4-(trifluoromethyl)aniline) were detected in the order of decreasing frequency. Cessna and Elliott (2004) investigated the seasonal variation of herbicide concentrations in farm dugout waters. Water samples were collected from dugouts before snowmelt in mid-February, during snowmelt runoff in April and May, and after snowmelt between May and October. This study found that during the three-year period of sampling, herbicides detections were most frequent during the spring application period and in late fall following dugout turnover. MCPA, 2,4-D, trifluralin, dicamba, diclofop, and clopyralid (3,6-dichloropyridine-2-carboxylic acid) were some of the herbicides detected in this study. The authors also noted that, of 181 water samples analyzed, herbicide concentrations in 47 % of them

exceeded European Union drinking water guidelines (0.03 to 0.10 μg/L for individual pesticides and 0.50 μg/L for mixtures of pesticides (EU, 1998)). Donald et al., (1999) also investigated herbicide occurrence in prairie wetlands. Nine herbicides were detected in water from 51 wetlands in Saskatchewan. MCPA, 2,4-D, and triallate were the most commonly detected herbicides. Herbicides have also been detected in precipitation. Various levels of 2,4-D, MCPA, bromoxynil, and dicamba, for example, were detected in prairie rainfall monitored from 1999 to 2001 in Alberta (Hill et al., 2002; 2003).

In a recent study (Donald et al., 2007), 27 herbicides (16 acidic, 6 neutral and 5 sulfonylurea) were detected in water collected from 15 drinking water reservoirs across the three prairie provinces (Alberta, Saskatchewan, and Manitoba). The study also found that 2,4-D, MCPA, clopyralid, dicamba, diclorprop (2-(2,4-dichlorophenoxy)propanoic acid), mecoprop (2-(4-chloro-2-methylphenoxy)propanoic acid), and bromoxynil (in the order of decreasing frequency) were most frequently detected. Some chemical properties of these herbicides are listed in Table 2.1. Maximum concentrations of these seven herbicides ranged from 83.1 to 1850 ng/L. The authors also noted that the water treatment facilities of this region reduced herbicide concentrations but did not remove them completely. The herbicides commonly detected in all the above studies coincide with list of most commonly used herbicides in the Prairie Provinces during 2003 (Waiser and Holm, 2005).

Due to their location within a semi-arid ecosystem, water levels in most prairie wetlands are driven by evaporation or evapo-transpiration (Hayashi, 1996). The possibility therefore exists for herbicide concentration as a result of evaporative water loss, a process which could exacerbate their effects on aquatic flora and fauna. Prairie wetland basins are also hydrologically closed, there are no stream outflows or inflows and water exchange with groundwater is minimal

(Hayashi et al., 1998). Consequently, whatever gets into these systems stays there and may be modified (biotically or abiotically) *in situ*.

2.2 Herbicides

2.2.1 Classification Based on Mode of Action

Herbicides are chemical compounds many of which mimic the chemical structure, functional group(s) or physiological function of naturally occurring plant compounds. For example, MCPA resembles the plant hormone auxin in its function and hence is called a synthetic auxin. Similarly, glyphosate (2-(phosphonomethylamino)acetic acid) is a derivative of the amino acid glycine. Herbicides can be classified into the following groups based on their mode or site of action.

2.2.1.1 Lipid synthesis (ACCase) inhibitors

Lipids are key components of plant cell membranes, cuticles, and suberin surrounding the endodermal cells of the Casparian strip. They also serve numerous functions in the plant, for example, as energy and signal molecules within the cell (Taiz and Zeiger, 2006). Any herbicide that inhibits lipid formation in plants can cause significant harm and lead to plant death. The site of action for these inhibitors is the fatty acid synthesis pathway which occurs in plant plastid stroma. These herbicides block the conversion of acetyl-CoA to malonyl-CoA by inhibiting the activity of acetyl-CoA carboxylase (ACCase) (Gronwald, 1991). This inhibition blocks production of phospholipids, used in building new cell membranes required for cell growth. Aryloxyphenoxypropionates (diclofop, fenoxaprop (2-[4-[(6-chloro-1,3-benzoxazol-2-yl)oxy]-phenoxy]propanoic acid), fluazifop (2-[4-(5-trifluoromethyl-2-pyridyloxy)phenoxy]propionic acid), quizalofop(2-[4-(6-chloroquinoxalin-2-yloxy)phenoxy]propionic acid) and cyclohexanedi-ones (sethoxydim (2-[1(ethoxyimino)butyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-

one), tralkoxydim (2-cyclohexen-1-one, 2-[1-(ethoxyimino)propyl]-3-hydroxy-5-(2,4,6-trimethylphenyl) are examples of this herbicide group.

2.2.1.2 Amino acid synthesis inhibitors

Amino acids are the building blocks of proteins. There are 20 amino acids synthesized in different plant pathways. Herbicides that interrupt any of these pathways prevent the synthesis of one or more essential amino acids. This group of herbicides can be divided into two classes: inhibitors of aromatic amino acids and branched chain amino acids.

Herbicides that block synthesis of the aromatic amino acids (tryptophan, phenylalanine and tyrosine) act on the shikimic acid pathway. They inhibit 5-enolpyruvyl shikimate-3-phosphate synthase (EPSP synthase) thereby blocking condensation of shikimate-3-phosphate and phosphoenolpyruvate to enolpyruvyl shikimate phosphate (EPSP) (Amrhein et al., 1980; Boocock and Coggins, 1983; Hollander-Czytko and Amrhein, 1987). The shikimic acid pathway is also important for the synthesis of flavenoids, lignins, and anthocyanins (Anderson, 1996; Huynh et al., 1988; Stryer, 1995). Without these essential biomolecules, the plant dies. Glyphosate is an example of an aromatic amino acid inhibitor.

Herbicides that inhibit the synthesis of the branched chain amino acids, isoleucine, leucine, and valine, block the activity of acetolactate synthase (ALS) and acetohydroxy synthase (AHAS). In doing so, they prevent conversion of α-ketoglutarate to 2-acetohydroxybutyrate and pyruvate to 2-acetolactate in the plant chloroplast (Anderson, 1996; Stryer, 1995). Imazamox (2-(4-Isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-5-methoxymethylnicotinic acid), chlorimuron (2-(4-chloro-6-methoxypyrimidin-2-ylcarbamoylsulfamoyl)benzoic acid), chlorsulfuron (2-chloro-*N*-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide), and thifensulfuron (3-(4-methoxy-6-methyl-1,3,5-triazin-2-ylcarbamoylsulfamoyl)thiophene-2-carboxylic acid) are some examples of this herbicide group.

2.2.1.3 Auxin-type herbicides (growth regulators)

Auxins are a class of naturally produced plant hormones (growth regulators). These compounds regulate many processes in plants, including cell growth and differentiation. Indole-acetic acid (IAA) is an example of a naturally occurring auxin. Auxin-type herbicides mimic IAA; hence, they are called synthetic auxins. Due to their auxin-type properties, these herbicides initially cause an increase in plant growth but later cause death, a phenomenon similar to hormesis (Stebbing, 1982) in which toxic substances may, at low, sub-lethal concentrations, have stimulating effects on organisms, especially on growth (Brock et al., 2000). Increased RNA polymerase activity, protein synthesis, ethylene production, cell wall loosening and enlargement, uncontrolled cell division and growth, and vascular tissue plugging are some of the responses in plants due to auxinic herbicides. MCPA, 2,4-D, dichlorprop, mecoprop, dicamba, and clopyralid belong to this group (Devine et al., 1993; Ross and Carole, 1999).

2.2.1.4 Inhibitors of pigment synthesis (bleaching herbicides)

Pigments are compounds that absorb light at certain wavelengths of the visible spectrum. Chlorophyll, for example, absorbs blue and red regions of the spectrum. Carotenoids are auxillary pigments associated with chlorophyll and they participate in an energy-transfer process which protects chlorophyll by dissipating the oxidative energy of singlet oxygen (Taiz and Zeiger, 2006). Bleaching herbicides inhibit the formation of carotenoids resulting in photo-destruction of chlorophyll and the bleaching of plant tissue. They inhibit pigment synthesis by inhibiting a key enzyme in the mevalonic acid pathway, phytoene desaturase (PDS), which is needed in carotenoid production (Tomaso, 1994). A few herbicides of this group, however, inhibit pigment synthesis by inhibiting the formation of diterpene and tetraterpene intermediates in the mevalonic acid pathway (Devine et al., 1993). Diterpenes and tetraterpenes are precursors to gibberellins and carotenoids, respectively. Gibberellins are plant growth hormones that

regulate developmental processes like stem elongation, seed germination, flowering and fruit senescence. Phytol, a component of the chlorophyll molecule, is also a diterpene (Taiz and Zeiger, 2006). Norflurazon (4-chloro-5-(methylamino)-2-(3-trifluoromethylphenyl)pyridazin-3(2H)-one) and fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1H)-pyridinone) are examples of herbicides inhibiting PDS enzyme activity. Clomazone (2-(2-chlorobenzyl)-4,4-dimethyl-1,2-oxazolidin-3-one) inhibits diterpene and tetraterpene synthesis. Amitrole (3-amino-1,2,4-triazole) is another example of a herbicide inhibiting pigment synthesis but it is a weak inhibitor of carotenoid synthesis. It is speculated that amitrol has several modes of action resulting in the accumulation of phytoene (a precursor of carotenoids), carotenes, lycopene, and phytofluene (Ross and Carole, 1999; Tomaso, 1994).

2.2.1.5 Photosynthesis inhibitors

These herbicides interrupt the energy production step or light reactions of photosynthesis and can be divided into two groups: Photosystem I (PS I) and photosystem II (PS II) inhibitors. Herbicides that inhibit PS I are also known as contact herbicides or membrane disrupters. These inhibitors disrupt the proper flow of electrons in the electron transport system (ETS) by diverting electrons from an iron-sulfur protein electron acceptor to the positively charged herbicide molecule. This process results in the formation of a free radical, which is subsequently re-oxidized (auto-oxidation) in the presence of oxygen and water producing the original cationic herbicide and superoxide anion radicals (O_2^-). These radicals are further altered to form hydroxyl radicals (•OH). Subsequent interaction of these unstable products with membrane fatty acids causes significant membrane disruption (Stryer, 1995; Tomaso, 1994). Paraquat and diquat are examples of this group.

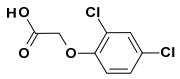
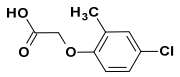
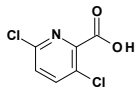
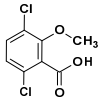
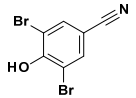
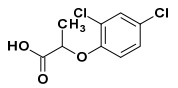
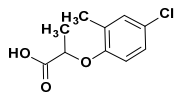
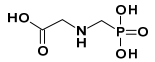
Herbicides that inhibit PS II bind to quinone, an ETS electron acceptor, and prevent the flow of electrons to plastoquinone (Tomaso, 1994). Bromoxynil, atrazine (2-chloro-4-(ethylamino)-6-

(isopropylamino)-*s*-triazine), metribuzin (4-amino-6-*tert*-butyl-4,5-dihydro-3-methylthio-1,2,4-triazin-5-one), and diuron (*N*-(3,4-dichlorophenyl)-*N*,*N*-dimethyl urea) are some examples of this group.

2.2.1.6 Seedling growth inhibitors

Although the specific mode of action of these herbicides is still unknown, inhibition of fatty acid and lipid biosynthesis has been observed. Synthesis of gibberellins and flavenoids was also found to be inhibited by this group of herbicides (Tomaso, 1994). Metolachlor (2-chloro-*N*-(2-ethyl-6-methyl-phenyl)-*N*-(1-methoxypropan-2-yl)acetamide), alachlor (2-chloro-*N*-(2,6-diethyl)phenyl-*N*-methoxymethylacetamide), and trifluralin are some examples of this group.

Table 2.1 Common name, chemical structure and class, IUPAC (International Union of Pure and Applied Chemistry) name, molecular weight and water solubility (at 25 ^{o}C) of the eight frequently detected and most commonly used herbicides in prairies.

Herbicide	Chemical Structure	Chemical Class	IUPAC Name	Molecular Weight	Water Solubility (mg/L)
2,4-D		Phenoxy acid	2-(2,4-dichlorophenoxy)acetic acid	221.04	900
MCPA		Phenoxy acid	2-(4-chloro-2-methylphenoxy)acetic acid	200.62	825
Clopyralid		Pyridine acid	3,6-dichloropyridine-2-carboxylic acid	192.00	7850
Dicamba		Benzoic acid	3,6-dichloro-2-methoxybenzoic acid	221.04	6500
Bromoxynil		Nitrile	3,5-dibromo-4-hydroxybenzonitrile	276.93	130
Dichlorprop		Phenoxy acid	2-(2,4-dichlorophenoxy)propanoic acid	235.10	620
Mecoprop		Phenoxy acid	2-(4-chloro-2-methylphenoxy)propanoic acid	214.60	620
Glyphosate		Glycine	2-(phosphonomethylamino)acetic acid	169.08	12000

2.2.2 Herbicide Fate in the Environment

Herbicides applied to cropland undergo various processes that are controlled by environmental factors. Volatilization (pre- and post-application) is the process by which herbicides change from liquid to a gaseous state. This process is governed by vapour pressure exerted by the gas when in equilibrium with the liquid state. When vapour pressure is higher so is the volatility. For soil-incorporated herbicides, volatilization facilitates herbicide movement into soil pore spaces; this process promotes distribution and increases efficacy. Volatilization can also be disadvantageous because of herbicide loss to the atmosphere and subsequent effects on non-target organisms (Anderson, 1996; Schaeffer and Anderson, 2007). A general rule for herbicide application is that if the vapour pressure is > 0.013 pascal at 25 ^{o}C, it will need to be incorporated into the soil to prevent volatilization loss. Trifluralin, for example, which has high volatility must be applied to the soil and incorporated by watering (Anderson, 1996).

Photodecomposition is the breakdown of herbicides by sunlight. If a herbicide absorbs high energetic UV light (< 400 nm), chemical bonds break and the molecular structure can change resulting in photodegradation products with reduced herbicidal activity. All herbicides are susceptible to photodecomposition. Most are white and absorb light in the range of 220 – 324 nm but dinitroaniline herbicides are yellow and absorb light at 376 nm, near the upper end of the UV range. Thus, these herbicides along with *s*-triazine and urea herbicides are more susceptible to photodecomposition (Anderson, 1996; Ross and Carole, 1999).

Leaching, the downward or lateral movement of herbicide in soil, may result in groundwater contamination. The amount leached is dependent on herbicide solubility, dissipation rate, and the sorptive capacity of soil. Soil pH, texture and permeability, rainfall and climatic conditions are other environmental factors that may influence leaching. Grover (1977) investigated leaching of

dicamba, picloram and 2,4-D in Canadian prairie soils and found that these three herbicides leached more readily in soils with pH of 7.5 and above.

Microorganisms have been shown to possess the necessary enzymes to mineralize complex organic compounds. Not surprisingly, various soil microorganisms like bacteria and fungi can degrade herbicides. Oxygen is required for herbicide breakdown with CO_2 released as a by-product. Dehalogenation (removal of halogen), dealkylation (removal of alkyl group), amide hydrolysis (formation of a carboxylic acid), ester hydrolysis (cleavage of ester bond), beta-oxidation (removal of 2 carbon atoms), and ring cleavage are other microbial decomposition reactions by which herbicides may be broken down. Soil moisture, aeration, temperature, pH, organic matter, and soil nutrients are environmental factors affecting microbial decomposition of herbicides. High nutrient and organic matter of soil and soil pH > 5.5 have been found to promote microbial decomposition of organic compounds like herbicides (Ross and Carole, 1999).

Adsorption is the association of herbicides with soil particles. Organic matter has strong negative charges and attracts cationic herbicides. Adsorbed herbicide will not be available for weed control. Strong adsorption also lowers the potential for leaching. The complex and heterogeneous nature of environmental matrices makes it difficult, if not impossible, to identify specific sorption mechanisms for most solid-chemical combinations and in most situations, several mechanisms like physical adsorption, hydrogen bonding, formation of coordination complexes, chemical adsorption may operate simultaneously (Delle Site, 2001). Sorption of herbicides to soil particles can promote transport of herbicides via wind erosion to farther distances from the site of application. Wind erosion is not only an economic loss due to loss of herbicide and soil but may cause toxic effects on the non-target organisms (Anderson, 1996;

Ross and Carole, 1999). The extent of sorption of organic contaminants to sediment has a major influence on its transport and fate in the environment (Chiou et al., 1998) and the risk of leaching and the extent of contamination of chemicals into groundwater or to surface waters (ter Laak et al., 2006).

2.3 Effects of Herbicides and Herbicide Mixtures on Non-Target Aquatic Organisms

2.3.1 Herbicide Mixtures

Herbicide mixtures in aquatic environments may exert adverse effects in four possible ways: i) simple concentration addition of their toxicity, ii) a more complex synergism in their mode of action, iii) a decreased activity (antagonism) and iv) no change in toxicity (independent action). According to the concept of 'concentration addition', a simple addition of concentrations of different components is reasonable for a mixture of chemicals with similar modes of action such that the total effect of the mixture can be reasonably predicted based on additive toxicity (Backhaus et al., 2000, 2004; Faust et al., 2000, 2001, 2003).

Herbicide mixtures are applied to farm fields to control diverse weed species (which have varying sensitivities) thereby increasing efficacy in controlling weeds. Not surprisingly then, they are often detected as mixtures in various environmental compartments (air, water and soil / sediment). The herbicide mixtures found in the environment are not limited to tank mixtures that are applied to farming fields but also consist of those applied in differing seasons or to differing geographic areas. Aquatic organisms, consequently, are typically exposed to numerous chemicals simultaneously or in sequence (Faust et al., 1994). When applied as part of a mixture, the toxic effect of the herbicide may or may not be similar to that shown by the individual herbicide. Unfortunately, most of the existing toxicological information relates to effects of single, pure substances, not to mixtures (Faust et al., 2000). The components of a mixture may exert synergistic or antagonistic affects compared to pure substance on an aquatic organism. It is

practically impossible to investigate the toxic effects of all potential combinations of chemicals in the aquatic environment. This task may be achieved by studying the most commonly occurring mixtures or developing toxicity models that can give adequate information for screening or regulatory purposes.

2.3.2 Predicting the Toxicity of Herbicide Mixtures

Safety guidelines for toxic substances are developed based on data generated from single chemical, single species experiments. NOAELs (no observable adverse effects level) or TDI (tolerable daily intake), for example, are developed using single species experimental data and considering a safety factor. Many of those single species experiments do not account for synergism or antagonism of toxic substances as well as interactions between species nor with abiotic factors. Living organisms, however, are exposed to multiple toxic chemicals in the environment simultaneously. This raises questions regarding existing safety guidelines for herbicides. Do they safeguard living organisms against multiple toxic chemicals even when each of those is present within the prescribed safety limits? Are the toxic actions of individual chemicals additive, synergistic or antagonistic? Although generating data for all toxic-chemical combination mixtures is not a feasible task, measures are needed to be taken to understand effects of these mixtures on non-target organisms.

There are two alternative concepts which predict the joint action of mixtures: concentration addition (CA) and independent action (IA) (Faust et al., 2000). Loewe and Muischnek (1926) developed the concept of CA and later Anderson and Weber (1975) introduced this concept into aquatic toxicology (Backhaus et al., 2000; Faust et al., 1994, 2000). The concept is suitable for the prediction of the toxicity of mixtures of similarly acting substances and is based on the idea that the components of a given mixture have a common site of primary action or act on the same molecular site (Backhaus et al., 2000). The total effect of the mixture can be reasonably

predicted based on the CA of the similarly acting chemicals (Backhaus et al., 2000, 2004; Faust et al., 2000, 2001, 2003). Mathematically, the CA concept is expressed as follows (Faust et al., 1994):

$$C_1 / ECx_1 + C_2 / ECx_2 = 1 \qquad \text{Equation 2.1}$$

where, C_1 and C_2 are the concentrations of the individual components 1 and 2, respectively, in a mixture which elicits the effect x; and ECx_1 and ECx_2 are the effect of each component 1 and 2, respectively, alone which elicit quantitatively the same effect x as the mixture (eg. EC_{50}).

In 1939, Bliss developed the concept of IA also known as response addition (Bliss, 1939). Bliss independence or effect multiplication assumes dissimilar modes of action of mixture components. This concept is based on the assumption that the components of a given mixture act on different physiological systems or different molecular sites leading to different physiological response (Backhaus et al., 2000; Faust et al., 2003). Thus, components of a mixture with different modes of action can cause effects at different sites in a single organism. Mathematically, IA concept is expressed as follows (Backhaus et al., 2000):

$$E(C_{mix}) = E(C_1 + ... + C_N) = 1 - \prod_{i-1}^{n} [1 - E(C_i)] \qquad \text{Equation 2.2}$$

where, $E(C_{mix})$ denotes the predicted effect on a scale 0 to 1 of an n-compound mixture, C_i is the concentration of the ith compound, and $E(C_i)$ is the effect of that concentration if the compound is applied singly.

Aquatic toxicologists have concluded that most chemicals acting with a similar mode of action exert joint effects on fish and other higher forms of life by simple addition (Faust et al., 1994; Nirmalakhandan et al., 1997). Faust et al. (1994) evaluated toxicity of 38 binary mixtures of 12 pesticides to algae and found that 66 % of the investigated mixtures showed toxicities as

predicted by CA, 24 % were less than that predicted by concentration addition and only 10 % were more toxic than expected by CA. In spite of these developments in understanding toxicity of mixtures, there is a need for more research on how mixtures of chemicals affect individual organisms or communities (Faust et al., 2000, 2003; Relyea, 2005, 2009).

2.3.3 Effects on Microbial Communities

Microbial communities consisting of algae and bacteria are vital members at the base of the wetland food web, where they play significant roles in biogeochemical cycles (carbon, nitrogen, phosphorus, and sulfur cycling), degradation, and decomposition (DeLorenzo et al., 2001). With the vast number of species of bacteria, algae, fungi and protozoa worldwide and there is a considerable diversity in the sensitivity of these microorganisms to herbicides (DeLorenzo et al., 2001). The majority of microbial toxicity studies have investigated the effects of herbicides on algae (Åkerblom, 2004). DeLorenzo et al. (2001) reviewed the literature on pesticide toxicity to aquatic microorganisms and stated that the mechanism of pesticide action in microbial species may not be the same as for the target organisms. In microorganisms, pesticides have been shown to interfere with respiration, photosynthesis, and biosynthetic reactions as well as cell growth, division, and molecular composition. Among the effects studies on microorganisms due to herbicides, atriazine is the most extensively tested compound.

Similar to higher organisms, microbial communities may metabolize, mineralize, detoxify or accumulate herbicides following exposure to them. Toxicity of individual herbicides to algae, cyanobacteria and duckweed was tested at expected environmental concentrations by Peterson et al. (1994) (Table 2.2). Results from this study indicated that there were considerable differences in sensitivity of various algal species to herbicides. Some algal and cyanobacterial species were inhibited while few others were stimulated at the same herbicide concentration. Relyea (2005) investigated the impact 2,4-D and glyphosate on the biodiversity and productivity of aquatic

communities. In this study, glyphosate at 3.8 mg/L reduced species richness (included were insects, tadpoles, snails, and zooplankton) by 22 % while 2,4-D at 0.12 mg/L had no effect.

Using chlorophyll *a* (Chl *a*) as an indicator of biomass of the primary producers occupying the base of the food chain, Relyea (2009) found suppression of Chl *a* in pond mesocosms treated with a mixture of five herbicides, acetochlor, metolachlor, glyphosate, 2, 4-D, and atrazine. Each herbicide was applied at 10 ng/L and results indicated that, Chl *a* in the mixture treatment was significantly lower than the control or individual herbicide treatments, except acetochlor. These results demonstrate that a single application of a herbicide mixture, even at low concentrations of individual herbicides, can have effects on algal communities in aquatic ecosystems.

Table 2.2 Toxicity of herbicides applied at expected environmental concentrations to algae, cyanobacteria, and duckweed[†].

Test organism	**Percent inhibition**[*]						
	2,4-D (2.92 mg/L)	**MCPA (1.40 mg/L)**	**Picloram (1.76 mg/L)**	**Bromoxynil (0.28 mg/L)**	**Atrazine (2.67 mg/L)**	**Chlorsulfuron (0.02 mg/L)**	**Triasulfuron[§] (0.02 mg/L)**
Algae							
Cyclotella	0	-3	-12	6	97	-8	13
Nitzschia	1	-18	-7	-40	99	-6	-39
Scenedesmus	-1	1	-7	-11	96	-3	-8
Selenastrum	-2	-18	-2	14	99	-13	-3
Cyanobacteria							
Microcystis spp. 1	9	0	3	0	96	-1	-15
Microcystis spp. 2	11	8	-27	-6	84	-23	-10
Oscillatoria	4	-7	8	-11	87	-17	8
Pseudoanabaena	-7	19	15	24	91	-2	1
Anabaena	-14	-15	14	-12	65	-4	15
Aphanizomenon	0	11	0	5	97	4	-13
Duckweed							
Lemna	34	42	10	-4	95	86	91

[†] Modified from Peterson et al. (1994).

[*] Values are presented as mean % inhibition of ^{14}C uptake for algae and cyanobacteria and 7-day growth for duckweed. Negative values indicate stimulation compared to the controls.

[§] 1-[2-(2-chloroethoxy)phenylsulfonyl]-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)urea.

It is important to understand not only the effects of herbicide mixtures but also the inter-trophic interactions. Cole et al. (1988) found a significant inter-dependence between bacterial production and phytoplankton primary production in the euphotic zones of lakes and the ocean. Soluble organic carbon excreted by phytoplankton is important for regulating bacterial growth in aquatic ecosystems (Cole et al., 1982). Any undesirable effects on such tightly knit lower trophic level members of the food web, therefore, may resonate to higher trophic levels, leading to instability in the ecosystem. Although some research has been done on toxicity of herbicide mixtures to microorganisms in aquatic ecosystems, it is quite limited. Most microbial studies have focused on herbicide degradation but not on their toxic effects (DeLorenzo et al., 2001).

Herbicides are also toxic to invertebrates such as *Daphnia* spp. The LC_{50} values in *Daphnia* for 2,4-D, dichlorprop, and clopyralid are >25 mg/L, 558 mg/L, and 225 mg/L, respectively, while EC_{50} values for dicamba and mecoprop are 34.6 mg/L and > 91 mg/L, respectively, and no observed adverse effects concentration (NOAEC) for MCPA is 11 mg/L (Patrick and Fallonsbee, 2004; USEPA, 2004, 2005, 2006, 2007a, 2007b, 2007c). Glyphosate has a LC_{50} value in Daphnia of 780 mg/L. Bromoxynil toxicity on *Daphnia* is dependent on the chemical form of the herbicide, for example, ester form of bromoxynil causes reproductive impairment in including daphnia at levels greater than 2.5 μg/L (> 2.5 ppb) and has EC_{50} 11-96 μg/L (11-96 ppb) (USEPA, 1998b).

2.3.4 Effects Studies

The Pest Management Regulatory Agency (PMRA) under Health Canada regulates registration of pesticide uses after thorough scientific evaluation of pesticide usage risks to the environment as well as public health. Similarly, this process is regulated by Environmental Protection Agency (EPA) in United States. Most herbicide uses have been registered using data

obtained from lower-tiered single-species laboratory tests. Risk assessment of these chemicals is based on the data generated from single-species laboratory tests (Fairchild et al., 1994). The LD_{50} value is a standardized and a common way of documenting toxicity. A chemical with a lower LD_{50} value is more acutely toxic than one with a higher LD_{50}. Similarly, LC_{50} (lethal concentration) is also a measure of acute toxicity. It is the concentration of a chemical that kills 50 % of the animals tested. Acute toxicity testing is carried out with a single dose and for a short period of time (usually less than 7 days). Chronic toxicological testing does not have any particular standardized testing procedure. However, acute tests are modified to accomplish chronic test conditions where lower and multiple doses for a longer duration (usually longer than 7 days and up to 3 months or longer) are employed. Acute tests usually measure the mortality rate while chronic tests measure sub-lethal effects like growth and reproduction. A single species (daphnia, chlorella, fish, rats, mice, rabbits, guinea pigs, and hamsters) is used as subjects in acute and chronic toxicity testing. These tests are usually performed in the laboratory under a controlled environment and responses are usually less variable. This enables the detection of smaller differences in endpoints between the control and treated groups with greater ease (USEPA, 1998a). However, the controlled environmental conditions may limit the range of responses. Alternative food sources, predator-prey stress, abiotic stress, etc., are usually not mimicked in the laboratory tests. Thus, responses in laboratory tests may not reflect those in the natural conditions. It is a huge scientific challenge to predict the behaviour of a population or communities in ecosystem based on the response of a single species in a controlled environment. Relationship between organism survival and ecosystem integrity is extremely complex and poorly understood (Cairns and Pratt, 1989; Cairns et al., 1992), making it difficult to replicate the conditions in laboratory experiments that prevail in the natural ecosystem.

Field observational studies measure biological changes in naturally occurring environments and usually involve more than one stressor. Exposure to stressors and effects of such exposure on organisms are more realistic and better applicable from field studies than those generated from laboratory tests or theoretical models. Field studies also permit observation of secondary effects as well as the recovery from the exposure to the stressor of the particular species and the community (USEPA, 1998a). Because conditions are not controlled, there is a chance of huge variability and a lack of sensitivity to detect small differences in effects. Another limitation is the difficulty in replicating field studies because biotic and abiotic factors are extremely diversified and vary constantly both in time and space (Boudou and Ribeyre, 1997). An ecosystem is a single entity with numerous living organisms at different trophic levels, complexed with interrelationships between the living organisms and their surroundings. Ecosystems are complex in terms of their biological organisation and diversity. According to Seitz (1994), *"the complexity and the individual history of each ecosystem give them unique properties which are not duplicated at another place and in many cases not even at the same place at different times"* as quoted by Boudou and Ribeyre (1997). Ecosystems are dynamic with multidirectional interactions between abiotic and biotic factors. In ecological testing, it is important to understand each component of the ecosystem to mitigate adverse effects caused by chemicals or factors xenobiotic or foreign to the ecosystem. Normal functioning and natural evolution of an ecosystem rely on the dependence and interdependence of the various communities of living organisms and their interactions with the abiotic environment (Boudou and Ribeyre, 1997).

2.4 Approaches for Ecosystem-Based Effects Studies

It is extremely difficult to mimic natural systems. The difficulty in dealing with the part and the whole of an ecosystem is widely recognised in science (Odum, 1984). To bridge the gap between the natural ecosystem and the laboratory microcosms, Odum (1984) suggested the use

of mid-sized, bounded or partially enclosed outdoor experimental setups called mesocosms. Microcosms and artificial streams are other approaches for multi-trophic field studies.

A microcosm is usually 1 L or less in volume and contains a single or multiple test species with its natural biotic and abiotic environment. Even though there are different types of microcosms, these do not represent the ecosystem completely and the biotic and abiotic factors are usually modified to accommodate the objectives of the experiment (van den Brink et al., 2005). Waiser and Robarts (1997), for example, used microcosms to investigate the effect of triallate herbicide on bacterial production, metabolism, and numbers in the presence and absence of nutrients, nitrogen and phosphorus. Microcosms may not represent field conditions in various aspects, however, they can be modified and adapted to simulate natural conditions in a controlled manner in laboratory settings. Bioreactors, for example, can simulate river flow as well as accommodate natural microbial communities. Lawrence et al. (2004), for example, investigated the effects of nickel, nutrients, and oxygen level on structure and function of river biofilm communities. These small-scale microcosm experiments can incorporate various field conditions and, as well as, can be replicated adequately with reasonable resources. In another example, Cuppen et al. (2002) used micrososms to study chronic effects of the application of a mixture of the insecticides chlorpyrifos and lindane. The glass aquaria microcosms consisted of periphyton and invertebrates together with the natural sediments. Sediments served as inoculum for microorganisms, algae, and zooplanktons. Several other macroinvertebrates, characteristic of study site, comprising several taxonomic groups and various trophic levels were also introduced. Twelve microcosms were used to accommodate various treatment concentrations and controls.

Mesocosms are larger than microcosms in volume, usually 1 L to 1000 L and sometimes even up to 15000 L or larger (van den Brink et al., 2005) and can incorporate a bigger portion of the

ecosystem; for example, 0.1 ha, 1.5 m depth and 700 L water with bottom sediments, submerged vegetation (*Chara* sp. and *Naja* sp.), emergent species (*Typha* sp. and *Sagittaria* sp.) with fish population as test organisms (Fairchild et al., 1992). These mesocosms can be used to investigate a significant part of the population without altering their surroundings. Fairchild et al. (1994) used mesocosms to study the effects of a herbicide (atrazine) and insecticide (esfenvalerate) mixture on a fish population (*Lepomis macrochirus*), algae (*Chara* sp. and *Naja* sp.), and zooplankton. In this study, twelve mesocosms of approximately 600 – 850 L were employed. Relyea (2009) used 1000-L outdoor mesocosms to understand how mixtures of pesticides and individual pesticides, at low concentrations, affect aquatic communities. Test organisms including zooplankton, phytoplankton, periphyton, and larval amphibians were employed in these mesocosms. In another study, Tlili et al. (2011) used outdoor mesocosms to investigate the impact of chronic and acute pesticide exposures on periphyton communities. In this study, the authors simulated contamination scenarios characteristic of a vineyard watershed with three successive pulse treatments of pesticide mixture. The outdoor mesocosms are subject to the natural conditions such as seasonal variations in light, temperature, day length and water quality. Mesocosms are more natural, represent the ecosystem on various aspects and rarely are the organisms and abiotic conditions manipulated (van den Brink et al., 2005).

Artificial streams mimic rivers/ponds and are built of metal or other material. Natural materials like rocks and sand from river system are used. Stream flow rate is also mimicked using water pumps or an over head tank (Bothwell, 1985). Carder and Hoagland (1998) used the artificial stream approach to investigate the combined effects of alachlor and atrazine on benthic algal communities. A re-circulating artificial stream was built from a 114-L oval-shaped Rubbermaid plastic livestock tub. Water used was from Wahoo Creak, 1.5 km southeast of

Wahoo, Nebraska. Electric motors were used to adjust the water velocities. Benthic sediments from the Wahoo Creek served as inoculum for algal communities. Biovolume of viable algal cells, cell density, and relative abundance of six dominant taxa were observed as endpoints.

Semi-field testing systems such as microcosms, mesocosms, or artificial streams are excellent experimental units because they have characteristics of both laboratory- and field-based experimental setups (van den Brink et al., 2002). In these systems, like laboratory experiments, a few variables can be controlled. For example, water volumes, treatment concentrations, sampling organisms, can be modified to suit the experimental design. Such experiments are exposed to various atmospheric processes (evaporation, precipitation, erosion, wind, etc.) and ecological progression of communities (succession of species in the event of loss as a result of environmental disturbance) providing a realistic model ecosystem. These semi-field testing systems are considered as an experimental tool bridging the gap between controlled laboratory experiments and the variable and complex conditions in the field. They link true experimental reproducibility and ecological realism (Brock et al., 2000). Microcosms and mesocosms not only help in understanding the effect of contaminants at the community level but also give insights into the process of recovery from those ecological disturbances. The United States Environmental Protection Agency (EPA) requires simulated field studies in pond mesocosms as part of the registration requirements for uses of some newer pesticides (USEPA, 2007b). It is important to conduct experiments using higher levels of biological organization because it is generally accepted that higher levels of biological organization like communities and ecosystems possess properties that are not present at lower levels like populations (Cairns and Pratt, 1989; Cairns et al., 1992). Because of their complexity and the costs involved in conducting field studies, research studies are rarely conducted using microcosms, mesocosms or artificial streams.

However, these complex systems might have their greatest utility for assessing environmental risks due to high volume chemicals such as pesticides and veterinary medicinal products (van den Brink et al., 2005).

2.5 Further Research

The following chapter (CHAPTER 3) is published in the Journal of Environmental Quality (2012, doi:10.2134/jeq2011.0376). This chapter describes the mesocosm study in which effects of glyphosate and two herbicide mixtures on microbial communities were investigated. One of the herbicide mixtures in this study was investigated in increasing doses with environmentally relevant concentration (ERC) of each herbicide as the base concentration while the other herbicide mixture and glyphosate were investigated at 1000 times ERC.

3. EFFECTS OF GLYPHOSATE AND TWO HERBICIDE MIXTURES ON MICROBIAL COMMUNITIES IN PRAIRIE WETLAND ECOSYSTEMS: A MESOCOSM APPROACH[1]

Abstract

A multi-trophic outdoor mesocosm system was used to mimic a wetland ecosystem and to investigate effects of glyphosate as well as two herbicide mixtures on wetland microbial communities. The glyphosate concentration utilized was 1000 times the environmentally relevant concentration (ERC). One herbicide mixture consisted of six auxin-type herbicides (2,4-D, MCPA, clopyralid, dicamba, dichlorprop, mecoprop), each at 1000 times the environmentally relevant concentration. The second mixture was comprised of eight herbicides including the six auxin-type herbicides, as well as bromoxynil and glyphosate. For this mixture, a dose-response approach was used to treat mesocosms with the ERCs of each herbicide as the base concentration. Algal biomass and productivity, and bacterial productivity and numbers for both pelagic and attached communities, were measured at different times over a 22-day period. The experimental results indicate that the eight herbicide mixture, even at low concentrations, produced negative effects on microbial communities. Glyphosate on its own, suppressed algal biomass and productivity for the duration of the study in both pelagic and biofilm communities. Algal biomass and productivity, although initially depressed in the auxin-type herbicide treatment, was stimulated from Day 9 until experiment end. Due to their similar modes of action, the effects of this particular herbicide mixture appear to be a result of concentration addition.

[1] Reproduced with permission: Sura S., Waiser M.J., Tumber V.P., Lawrence J.R., Cessna A.J., Glozier N.E. (2012) Effects of glyphosate and two herbicide mixtures on microbial communities in prairie wetland ecosystems: A mesocosm approach. Journal of Environmental Quality 41:732-743.

Such negative effects, however, were short-term and microbial communities recovered from herbicide exposure. Based on evidence presented in this study, it appears that glyphosate has a higher potential to inhibit primary productivity and chlorophyll *a* content in pelagic and attached wetland algal communities than the auxin-type herbicide mixture.

3.1 Introduction

More than 6 % of the earth's surface (~ 8.6 million sq. km.) is covered by wetlands and these systems are found globally except in Antarctica (Mitsch and Gosselink, 2000; Ornes, 2008). Canada possesses 25 % of the world's wetlands, and 18 % of its landmass (> 127 million ha.) is covered by these water bodies. The Prairie pothole region (PPR) across south-central Canada and the north-central United States contains more than four million wetlands and ponds which cover 15 to 25 % of the landmass (Mitsch and Gosselink, 2000). The total wetland area in North America, however, has declined substantially, mostly due to human activities such as drainage (Dahl, 2000).

Prairie wetlands serve an important role in providing wildlife habitat, and water storage and filtration. One of the key geographical features of the PPR is that the wetlands are embedded among agricultural operations where herbicides, insecticides and fungicides are commonly used (Donald et al., 1999; Waiser and Robarts, 1997). These pesticides may eventually reach the surrounding wetlands via spray drift, aerial deposition, surface runoff, or ground water flow (Grover et al., 1988; Waite et al., 1992) and, consequently, are frequently detected in prairie wetlands (Donald et al., 1999, 2007; Waite et al., 2004) and farm dugouts (Cessna and Elliott, 2004). These studies revealed that the seven herbicides most commonly found in prairie waters (drinking water reservoirs, wetlands and farm dugouts) were: 2,4-D [2-(2,4-dichlorophenoxy)acetic acid], MCPA [2-(4-chloro-2-methylphenoxy)acetic acid], dicamba [3,6-dichloro-2-methoxybenzoic acid], clopyralid [3,6-dichloropyridine-2-carboxylic acid], bromoxynil [3,5-dibromo-4-hydroxybenzonitrile], dichlorprop [2-(2,4-dichlorophenoxy)-propanoic acid], and mecoprop [2-(4-chloro-2-methylphenoxy)propanoic acid].

Prairie wetland food webs consist of primary producers (free-living and attached algae, submerged and emergent plants), bacteria, benthic invertebrates, emergent insects and higher

trophic level mammals and migrating waterfowl. These aquatic ecosystems are key ecological features of the prairie region, supporting 50 to 80 % of the North American waterfowl population and approximately half of other migratory birds each year (Mitsch and Gosselink, 2000). Microbial communities consisting of phytoplankton and bacteria are vital members of the wetland food web, where they play significant roles in carbon, nitrogen and phosphorus cycling, as well as degradation of pesticides and decomposition of organic matter (DeLorenzo et al., 2001; Waiser and Robarts, 2004). Any stressor (such as a herbicide) which might affect production at this level may resonate to higher trophic levels; for example, zooplankton which feed on algae and ducks which feed on zooplankton and benthic invertebrates (Waiser and Holm, 2005). These effects are likely more pronounced with pesticide (herbicides, insecticides, fungicides) mixtures due to the variety of modes of action and therefore effects may be seen at multiple ecosystem trophic levels.

In this study, a multi-trophic outdoor mesocosm system was used to mimic the wetland ecosystem and to investigate effects of a mixture of eight herbicides on production and biomass of pelagic and attached wetland microbial communities (algae, bacteria) using a dose-response approach. Seven of the herbicides used were those detected most frequently in prairie aquatic ecosystems (Donald et al., 2007) and used widely in prairie crop production (Waiser and Holm, 2005). The mixture included 2,4-D, MCPA, dicamba, clopyralid, dichlorprop, mecoprop, bromoxynil, and glyphosate [2-(phosphonomethylamino)acetic acid]. Glyphosate is one of the most commonly and heavily used herbicides in Canada.

The intent of the current study was to provide much needed knowledge regarding effects of this environmentally relevant herbicide mixture on sensitive prairie wetland microbial communities. Such knowledge would, in turn, help regulatory agencies to protect prairie

wetlands when estimating risk due to pesticides and to develop strategies to mitigate adverse effects. In order to maintain and sustain the integrity of these important ecosystems, an understanding of herbicide mixture effects is imperative.

3.2 Materials and Methods

3.2.1 Study Site Description and Mesocosm Setup

Seven mesocosms were positioned adjacent to Pond 79 at the St. Denis National Wildlife Area (52^{o}02' N 106 ^{o}06' W) in late May 2007. This refuge is located 45 km east of Saskatoon, SK, in the Prairie Pothole Region (PPR) of Saskatchewan. Each mesocosm system consisted of one 183 by 61 cm circular plastic holding tank (Figure 3.1). Within these mesocosms, five 49 by 61 cm stainless steel tanks were installed and used by another research group to study mixture effects on emergent insects. The outer mesocosm walls were covered with reflective material while a shade cloth (200 by 200 cm) was positioned over each mesocosm approximately 100 cm above ground, to achieve water temperature and light saturation levels similar to adjacent ponds (Culp et al., 2003).

Water (1200 to 1300 L) containing microbial and zooplankton communities from Pond 79 was transferred into the plastic holding tank (mesocosm) and two-stroke submersible pumps circulated water from the holding tank into the five stainless steel tanks. Phytoplankton and zooplankton communities were allowed to develop in the mesocosms for about 30 days. Biofilm (attached) communities were grown *in situ* on round glass coverslips 2.54 cm^2 loaded onto PVC plates prior to deployment into each mesocosm (Figure 3.2). Tracks in the PVC plates held coverslips in place during incubation at a water depth of approximately 20 cm. PVC plates with coverslips were deployed 1 day prior to treatment. Water in each holding tank was treated with a mixture of commercially formulated herbicides (including associated adjuvants and surfactants). The herbicides used in this study along with their formulation concentrations and trade names

were 2,4-D (282 g/L, Nufarm Estaprop PLUS, Nufarm Agriculture), MCPA (500 g/L, Nufarm MCPA Amine 500, Nufarm Agriculture), clopyralid (360 g/L, Lontrel 360, Dow AgroSciences), dicamba (480 g/L, Oracle, Gharda Chemicals Ltd.), bromoxynil (280 g/L, Pardner, Bayer CropScience), dichlorprop (300 g/L, Nufarm Estaprop PLUS, Nufarm Agriculture), mecoprop (150 g/L, Mecoprop, United Agri-Products), and glyphosate (360 g/L, Glyphos, Cheminova).

Figure 3.1 Photograph showing mesocosms deployed in May 2007 adjacent to Pond 79 at St. Denis National Wildlife Area, Saskatchewan, Canada.

3.2.2 Application of Herbicides to Mesocosms

Herbicide treatments were applied to each mesocosm randomly according to Table 3.1 on 25th June 2007 (Day 0). The same proportional herbicide mixture was added to each mesocosm, but in increasing concentrations (dose-response additions). The base (1X) concentration was the environmentally relevant concentration (ERC) for each of the seven herbicides except for mecoprop. It was discovered later in the study that mecoprop was accidentally added twice to the stock solution resulting in twice the intended target concentration in all the mesocosms. The ERC value of a pesticide is defined as the concentration detected in water bodies as a result of run-off, spray drift, aerial deposition, and other processes. In this study, the ERC values were based on average concentrations calculated from those detected in wetland ecosystems across the

Canadian prairies (Donald et al., 1999, 2005, 2007 and other pesticide concentration data held by Environment Canada. Mesocosms 1X, 10X, 500X, and 1000X were treated with 1, 10, 500 and 1000 times the ERC of each of the eight herbicides, respectively. Mesocosm AUX was treated with 1000 times ERC of the six auxin-type herbicides (2,4-D, MCPA, clopyralid, dicamba, dichlorprop, and mecoprop), mesocosm GLY was treated with glyphosate at 1000 times its ERC and mesocosm CON served as a control. Calculated herbicide volumes for each treatment were mixed into approximately 10 L of wetland water and then transferred to a 15-L hand-operated sprayer. The mixture was uniformly injected into each mesocosm with the sprayer nozzle beneath the water surface. In spite of lack of treatment replication, water volumes in each mesocosm were large enough that a composite sample from different locations within each mesocosm would represent the mesocosm adequately. In addition, every effort was made to replicate the adjacent wetland, Pond 79, in these mesocosms. The intent of the dose-response approach was to provide experimental evidence of mixture concentrations relative to the $NOEC_{eco}$ (the highest concentration at which no effect is observed for the most sensitive endpoint studied in the ecosystem) and the $LOEC_{eco}$ (the lowest concentration at which an effect is observed for the most sensitive endpoint studied in the ecosystem). The effects of herbicide mixture at higher concentrations (500 and 1000 times ERC) provide experimental evidence for worst-case scenario.

Figure 3.2 Photograph showing PVC plates with coverslips deployed at a water depth of approximately 20 cm in each mesocosm. Biofilm growth on the coverslips can be noted in this photograph.

Table 3.1 Herbicide concentrations used to treat mesocosms at environmentally relevant concentrations (ERCs)[†].

Herbicides	**Guideline value[‡] (μg/L)**	**1X[§] (μg/L)**	**10X[¶] (μg/L)**	**500X[#] (μg/L)**	**1000X[††] (μg/L)**	**AUX[‡‡] (μg/L)**	**GLY[§§] (μg/L)**	**CON[¶¶] (μg/L)**
2,4-D	4	0.0788 (17.5)[##]	0.788	39.4	78.8	78.8	0	0
MCPA	2.6	0.0788 (17.5)	0.788	39.4	78.8	78.8	0	0
Clopyralid	–[†††]	0.0225 (5)	0.225	11.3	22.5	22.5	0	0
Dicamba	10	0.0225 (5)	0.225	11.3	22.5	22.5	0	0
Dichlorprop	4	0.00563 (1.25)	0.0563	2.81	5.63	5.63	0	0
Mecoprop[‡‡‡]	4	0.00563 (1.25)	0.0563	2.81	5.63	5.63	0	0
Bromoxynil	5	0.0113 (2.5)	0.113	5.63	11.3	0	0	0
Glyphosate	65	0.225 (50)	2.25	113	225	0	225	0

[†] The ERC value of a pesticide was defined as the concentration detected in water bodies as a result of run-off, spray drift, aerial deposition, and other processes.
[‡] Water quality guideline for the protection of aquatic life in freshwater (CCME, 1999).
[§] Environmentally relevant concentrations of all 8 herbicides (1X).
[¶] 10 times ERCs of all 8 herbicides (10X).
[#] 500 times ERCs of all 8 herbicides (500X).
[††] 1000 times ERCs of all 8 herbicides (1000X).
[‡‡] 1000 times ERCs of the six auxin-type herbicides (AUX).
[§§] 1000 times ERC of glyphosate (GLY).
[¶¶] No herbicide treatment, Control (CON).
[##] Values in the parenthesis are the relevant proportions of the respective herbicides in the total mixture.
[†††] No guideline established.
[‡‡‡] Mecoprop concentrations presented are intended target concentrations; however, it was accidentally added at twice these concentrations.

3.2.3 Pelagic and Biofilm Community Sampling

For nutrients and major ions, a composite water sample was collected from each mesocosm and Pond 79, every two weeks. For the pelagic community, a composite water sample from each mesocosm was collected on Days 1, 2, 3, 9, 16, and 22 into a 2-L amber PVC bottle. Water was subsequently screened through a 150-µm Nitex mesh (Dynamic Aqua-Supply Ltd., BC, Canada) to remove large zooplankton and then used to measure all variables. For the biofilm community, coverslips containing biofilms were harvested (n = 4 for each parameter measured) on Days 8 and 21.

3.2.4 Sample Analysis

3.2.4.1 Water quality parameters

Surface water temperature, specific conductivity, dissolved oxygen (DO), pH, and alkalinity were measured at the same time of the day (between 10:00 and 11:00 am) in each mesocosm as well as in the Pond 79, on each sampling date using a YSI 650MDS data display and logging unit connected to a 600XLM-0 multi-parameter water quality monitoring probe (YSI Inc., Ohio, USA). Nutrient analyses, including total phosphorus (TP), ammonium nitrogen (NH_4^+) (both analyzed using a Seal Colorimeter AA-3 (Seal Analytical, Norderstedt, Germany)), and dissolved organic carbon (DOC) (using a DOC analyzer, Tekmar-Dohrmann Phoenix 8000, Ohio, USA) were carried out according to established methods (EC, 1992). Major ions including calcium (Ca), magnesium (Mg), sodium (Na), and potassium (K) were measured using ion chromatography (ICS-1000, Dionex Canada Ltd., Oakville, ON, Canada) according to established methods (EC, 1992).

3.2.4.2 Pelagic community analysis

Primary productivity (PP) was determined using a standard light/dark bottle ^{14}C method (Wetzel and Likens, 1991). Volumetric rates of PP (mg C m^{-3} h^{-1}) were calculated from ^{14}C

incorporation rates and ^{12}C concentrations (from temperature, pH and alkalinity data) available to phytoplankton (Robarts et al., 1992). Daily rates were estimated by multiplying hourly rates by 10 to simulate 10 h of daylight (Cole et al., 1988).

Alkalinity was determined by end-point titration with 0.01N H_2SO_4 solution (Clesceri et al., 1998) using a TitraLab TIM850 titration system with SAC80 autosampler (Radiometer Analytical SAS, France) linked to TitraMaster 85 software.

Bacterial productivity (BP) was determined by the rate of incorporation of a radioactively-labelled nucleotide (^3H-thymidine) into bacterial DNA (Robarts and Wicks, 1989). BP rates were calculated from thymidine incorporation using a conversion factor for a eutrophic lake of 2.0 x 10^{18} bacterial cells produced per mole ^3H-thymidine (Bell et al., 1983; Coveney and Wetzel, 1988). A factor of 20 fg C per bacterial cell was then used to convert cell numbers to an estimate of carbon produced (Lee and Fuhrman, 1987; Reitner et al., 1999). Because the carbon content of most bacterial cells is in the 10 to 20 fg C $cell^{-1}$ range (Cotner and Biddanda, 2002), the upper limit was chosen to represent carbon content of bacterial cells in eutrophic ecosystems. Daily volumetric rates were estimated by multiplying hourly rates by 24 (Cole et al., 1988).

Bacterial numbers were estimated using the DAPI (4,6-diamidino-2-phenylindole) fluorescent DNA staining method (Porter and Feig, 1980). Four replicate 10-mL aliquots of screened water were pipetted into sterile Vacutainer tubes (VWR International, Mississauga, ON, Canada) and preserved with 200 μL of Lugol's solution at 4^o C until analysis. Bacteria were subsequently stained using DAPI and counted using epifluorescence microscopy (Waiser, 2001a). A minimum of 200 cells were counted for each replicate.

Phytoplankton biomass was estimated as chlorophyll *a* (Chl *a*) (Wetzel and Likens, 1991). Water samples were filtered through a 47-mm 1.2-μm pore-size Whatman GF/C filters in

replicates (n = 4). Chl *a* was extracted using a boiling ethanol technique and subsequently analysed fluorometrically using a Turner Design Model 10-AU digital fluorometer (Turner Designs, Sunnyvale, CA) (Waiser and Robarts, 1997).

3.2.4.3 Biofilm community analysis

For biofilm primary productivity, coverslips were randomly harvested and placed in crystallization dishes containing 20 mL of 0.2-μm filter sterilized water and 450 μL $NaH^{14}CO_3$. Dark control dishes were covered with foil and all were incubated *in situ* for one hour. Coverslips were then removed, individually placed in 50 mL Falcon tubes containing 50 mL 0.2-μm filter sterilized water, placed on ice and transported to the laboratory (Waiser, 2001b). Coverslips were crushed using a clean glass stirring rod, and contents subsequently filtered through 47-mm 0.45-μm pore-size Whatman cellulose nitrate filters under gentle vacuum. Filters were then treated and radioactivity was counted as noted above for pelagic PP. Biofilm PP rates were calculated as described above for pelagic samples except that rates were based on the area of the coverslip, not the volume filtered.

For biofilm bacterial productivity, each crystallization dish with a biofilm coverslip was fortified with ^{3}H-thymidine solution (334 μL, 20 nM) and incubated for 1 h. Killed controls received 2 mL formalin. At incubation end, each coverslip was placed in a centrifuge tube to which formalin (2 mL) and 5 N NaOH (2 mL) were added to stop the incubation (Waiser, 2001b). All coverslips were then crushed using a clean sterile glass rod and DNA extracted in 100 % TCA. ^{3}H-thymidine incorporated into bacterial DNA was then measured and BP was calculated as described earlier.

For phytoplankton biomass, four coverslips were harvested from each mesocosm and each placed in a 50-mL centrifuge tube containing 10 mL of 90 % ethanol. Chl *a* was extracted as described earlier.

3.2.4.4 Statistical analysis

A two-way repeated measured analysis of variance (RM ANOVA) was conducted on pelagic PP, BP, and Chl *a* data for all treatment levels over time. One-way ANOVAs and post hoc Tukey's tests were also performed on the same variables to detect whether 1X, 10X, 500X, and 1000X were significantly different from CON on Day 22 for pelagic data and on Days 8 and 22 for biofilm data. Additionally, a student's *t*-test was also performed to determine whether there were significant differences between AUX or GLY and CON on Day 22 for pelagic data and on Days 8 and 22 for biofilm data. The significance level was $p < 0.05$ and all tests were conducted using SAS statistical software package, version 9.1 (SAS Institute Inc., Cary, North Carolina, USA).

3.3 Results

3.3.1 Water Quality Parameters

Temperature, alkalinity, DO and DOC concentrations in each mesocosm on each sampling day were similar to Pond 79 ($p > 0.05$) (Table 3.2). Although specific conductivity and pH were similar across all mesocosms, they were below those in Pond 79 ($p > 0.05$) (Table 3.2). Nutrient and major ion concentrations measured on Days 0, 9, and 21 in each mesocosm were similar to Pond 79 ($p > 0.05$), with the exception of TP ($p < 0.05$) (Table 3.3). Although TP concentrations in all mesocosms were less than those in Pond 79, they were still adequate to support algal growth.

Table 3.2 Average values for various water quality parameters in all mesocosms and Pond 79.

Mesocosms[†] / Pond	**Temperature (^oC)**	**Specific Conductance (μS/cm)**	**DO[§] (mg/L)**	**pH**	**Alkalinity (mg/L $CaCO_3$)**	**DOC[¶] (mg/L)**
1X	16 ± 4	3840 ± 170	9.1 ± 1.9	8.4 ± 0.2	450 ± 20	44 ± 1
10X	16 ± 4	3930 ± 190	8.9 ± 1.9	8.4 ± 0.2	460 ± 20	46 ± 2
500X	16 ± 4	3870 ± 160	8.9 ± 1.8	8.4 ± 0.2	450 ± 20	43 ± 3
1000X	16 ± 4	3900 ± 130	8.9 ± 1.9	8.4 ± 0.2	460 ± 10	46 ± 5
GLY	16 ± 4	3850 ± 160	8.9 ± 2.1	8.4 ± 0.3	460 ± 10	46 ± 2
AUX	16 ± 4	3900 ± 150	9.1 ± 1.8	8.4 ± 0.2	450 ± 20	46 ± 2
CON	16 ± 4	3890 ± 190	9.1 ± 1.9	8.4 ± 0.2	450 ± 20	44 ± 3
Pond 79	18 ± 4	4240 ± 260*	7.1 ± 0.8	8.0 ± 0.3*	500 ± 40	49 ± 5

[†] Values for each parameter are averages of measurements on various (Days 0, 1, 2, 3, 9, 16, 22) sampling days ± standard deviations, n = 7, for each mesocosm and Pond 79.

[*] Average value with * symbol indicates significantly different from rest of the values in the column at 0.05 level of significance.

[§] Dissolved oxygen (DO).

[¶] Dissolved organic carbon (DOC).

Table 3.3 Nutrients (ammonium nitrogen (NH_4^+) and total phosphorus (TP)) and major ions (calcium (Ca), magnesium (Mg), sodium (Na), and potassium (K)) in mesocosms and Pond 79.

Mesocosms† / Pond	Nutrients (mg/L)		Major Ions (mg/L)			
	NH_4^+	TP	Ca	Mg	Na	K
1X	0.11 ± 0.01	0.15 ± 0.02	131 ± 11	347 ± 16	356 ± 22	52 ± 3
10X	0.12 ± 0.03	0.17 ± 0.03	139 ± 7	347 ± 34	364 ± 23	53 ± 3
500X	0.11 ± 0.04	0.18 ± 0.04	137 ± 7	354 ± 16	358 ± 15	52 ± 2
1000X	0.10 ± 0.03	0.17 ± 0.00	136 ± 7	344 ± 30	347 ± 31	50 ± 4
GLY	0.10 ± 0.03	0.19 ± 0.01	137 ± 3	337 ± 35	344 ± 34	52 ± 5
AUX	0.10 ± 0.02	0.14 ± 0.02	135 ± 11	351 ± 8	356 ± 18	52 ± 3
CON	0.11 ± 0.02	0.16 ± 0.02	136 ± 6	344 ± 28	348 ± 28	51 ± 4
Pond 79	0.10 ± 0.02	0.41 ± 0.09 *	147 ± 7	396 ± 55	393 ± 52	49 ± 6

† Values for each parameter are averages of measurements on 3 sampling days (day 0 (pretreatment), Day 9, and Day 21) ± standard deviation, n = 3, for each mesocosm and Pond 79).

* Average value with * symbol indicates significantly different from rest of the values in the column at 0.05 level of significance.

3.3.2 GLY vs CON

Pelagic PP rates were significantly different over time when GLY was compared to CON ($p < 0.05$) (Figure 3.3A). On Days 1 and 22, pelagic PP was significantly lower than CON. As well, in GLY, PP on Day 22 was higher than on Day 1.

Although average pelagic Chl *a* in GLY increased over the study period (Figure 3.3A) it was still significantly lower than CON on Day 22 ($p < 0.05$).

Pelagic BP was significantly different over time when GLY and CON were compared ($p < 0.05$) (Table 3.4). Generally, BP in GLY was lower than CON, especially during the first three days of the study. Bacterial numbers in GLY, however, were not significantly different (from CON ($p > 0.05$ - data not shown).

On average, biofilm PP in GLY was significantly lower than CON ($p < 0.05$) on Days 8 and 22 (Figure 3.3B). Although some increase in GLY PP over time was noted, PP here was the lowest of all herbicide treatments (Figure 3.3B, Figure 3.4B, and Figure 3.7). In contrast to the PP results, algal biomass was significantly lower in GLY compared to CON only on the last sampling day ($p = 0.0079$) (Figure 3.3B). For BP, although rates in CON were higher than GLY on both sampling days they were only significantly higher on Day 8 ($p < 0.05$) (Table 3.5). Average PP (Figure 3.3B) and BP (Table 3.5) in GLY on Day 21 were higher than those initially indicating some biofilm growth over time.

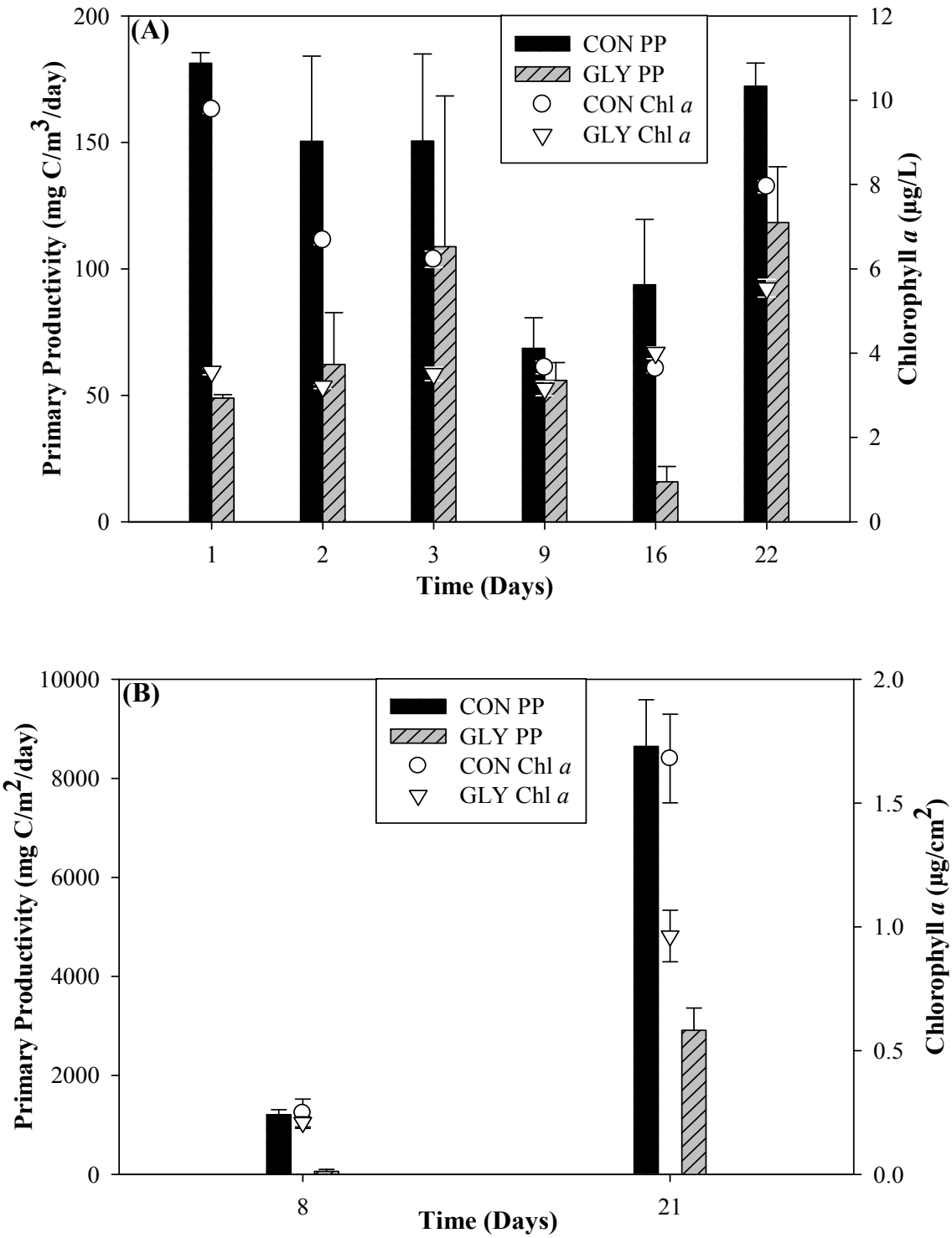


Figure 3.3 Primary productivity (PP) rates and chlorophyll *a* (Chl *a*) in CON (control) and GLY (glyphosate treatment at 1000 times environmentally relevant concentrations) mesocosms in (A) Pelagic community; (B) Biofilm community. Error bars represent standard deviation, n = 4.

Table 3.4 Pelagic bacterial productivity (BP) in all mesocosm treatments.

Mesocosm	Pelagic bacterial productivity (BP) (mg Carbon/m^3/day)[†]					
	Day 1	**Day 2**	**Day 3**	**Day 9**	**Day 16**	**Day 22**
1X*	175 ± 34	284 ± 13	174 ± 14	349 ± 13	201 ± 63	572 ± 49
10X*	154 ± 4.4	217 ± 32	151 ± 7.0	326 ± 74	245 ± 5.4	652 ± 70
500X*	176 ± 16	212 ± 49	155 ± 27	253 ± 48	246 ± 75	572 ± 76
1000X*	138 ± 4.6	232 ± 37	121 ± 27	321 ± 65	336 ± 41	505 ± 34
AUX*	140 ± 11	253 ± 24	155 ± 24	171 ± 59	219 ± 15	598 ± 61
GLY*	116 ± 9.7	131 ± 41	123 ± 9.8	303 ± 17	299 ± 15	408 ± 67
CON	190 ± 7.4	300 ± 36	195 ± 16	287 ± 23	279 ± 40	471 ± 44

[†] Values are reported as mean ± standard deviation, n = 4.

* Mesocosm treatment with * symbol indicates significantly different from CON (control) mesocosm at 0.05 level of significance.

Table 3.5 Biofilm chlorophyll *a* (Chl *a*) and bacterial productivity (BP) in all mesocosm treatments.

Mesocosm	Biofilm Chlorophyll *a* (Chl *a*) ($\mu g/cm^2$)†		Biofilm Bacterial Productivity (BP) (mg Carbon/m^2/day)†	
	Day 8	Day 21	Day 8	Day 21
1X	0.35 ± 0.06	1.3 ± 0.36	4.09 ± 0.62	4.55 ± 0.21
10X	0.16 ± 0.03*	0.86 ± 0.16*	4.34 ± 1.10	2.91 ± 0.56*
500X	0.10 ± 0.01*	0.86 ± 0.20*	4.11 ± 0.83	2.78 ± 0.86*
1000X	0.43 ± 0.04*	0.99 ± 0.14*	2.93 ± 0.65*	3.62 ± 0.10*
GLY	0.21 ± 0.02	0.96 ± 0.10*	2.98 ± 0.51*	4.11 ± 0.34
AUX	0.36 ± 0.03*	0.93 ± 0.20*	5.26 ± 0.81*	4.00 ± 0.11
CON	0.25 ± 0.06	1.69 ± 0.18	3.99 ± 0.31	4.24 ± 0.30

† Values are reported as mean ± standard deviation, n = 4.

* Values within the column with * symbol indicates significantly different from CON (control) mesocosm value at 0.05 level of significance.

3.3.3 AUX vs CON

Statistical analysis showed a significant effect of time on PP for AUX and CON ($p < 0.05$) (Figure 3.4A). Here, PP was higher than those in CON on Days 9 and 22 but similar on Days 3 and 16. Average PP in AUX on Day 22 was higher than that on Day 1.

Although Chl *a* was lower than CON on Days 1 and 2, higher biomass occurred from Day 9 until study end. By this time, algal biomass was significantly higher than CON ($p < 0.05$) (Figure 3.4A).

Generally, bacterial numbers in AUX were significantly lower than CON throughout the sampling period ($p < 0.05$) (data not shown). Similar results were noted for BP which was lower than CON during the whole sampling period except on Day 22 when it was significantly higher ($p = 0.02$).

Compared to CON, biofilm PP and Chl *a* on Day 8 in AUX were both significantly higher ($p < 0.05$), but then significantly lower by Day 21 ($p < 0.05$) (Figure 3.4B). Significantly higher PP and Chl *a* was noted in AUX when Day 21 was compared to Day 8 ($p < 0.05$) indicating some growth in the AUX biofilms had occurred.

Although biofilm BP in AUX was significantly higher than CON on Day 8 ($p = 0.02$), by Day 21, no difference was detected ($p = 0.24$) (Table 3.5).

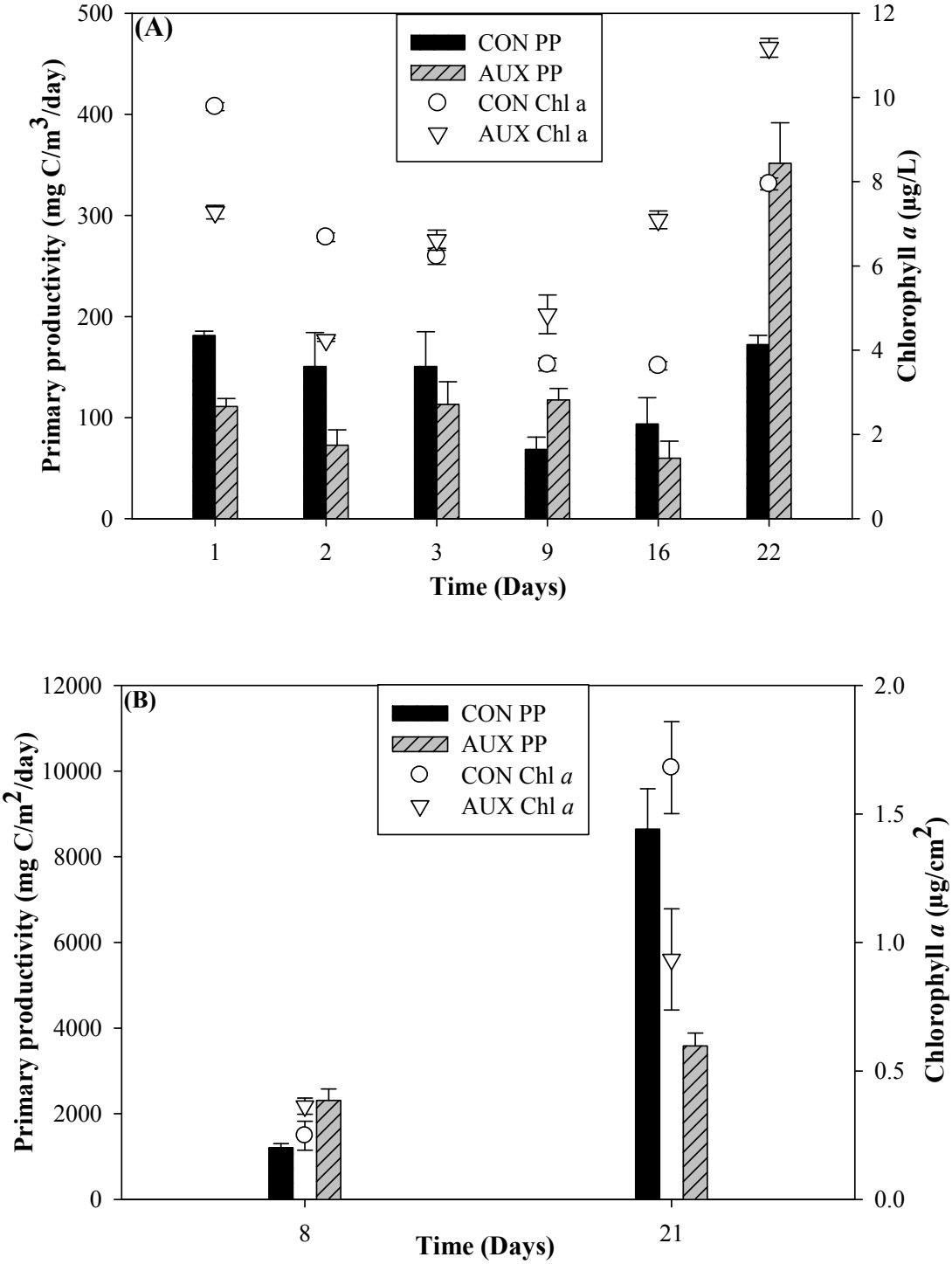


Figure 3.4 Primary productivity (PP) rates and chlorophyll *a* (Chl *a*) in CON (control) and AUX (six auxin-type herbicides treated at 1000 times environmentally relevant concentrations) mesocosms in (A) Pelagic community; (B) Biofilm community. Error bars represent standard deviation, n = 4.

3.3.4 1X, 10X, 500X, and 1000X vs CON

Pelagic PP across all treatments (1X, 10X, 500X, and 1000X) was significantly different over time ($p < 0.05$) compared to the CON (Figure 3.5). Although PP initially showed a dose-response relationship this relationship was not evident after Day 9. On Day 9, PP in 1X was higher than CON, lower than CON in 1000X, and similar to CON in 10X and 500X. By Day 16, PP across all treatments was similar to CON except 10X which was lower. On the last sampling day, PP across all treatments was similar to CON except 500X which was significantly lower ($p < 0.05$).

Chl *a* was significantly different over the study across all treatments ($p < 0.05$) (Figure 3.6). On Day 22, Chl *a* in 1X, 10X, and 500X treatments was significantly lower than CON whereas it was significantly higher than CON in 1000X ($p < 0.05$).

The results suggest that the pelagic BP rates were different over time for all the treatments ($p < 0.05$) (Table 3.4). BP in 1X was similar to CON on Days 1, 2, and 3 but lower than CON on Days 1, 2, and 3 in 10X. In 500X, BP was similar on Days 1 and 3, but lower than CON on Day 2. In contrast, BP in 1000X, was lower than CON on Days 1 and 3 but similar on Day 2. Pelagic BP rates on Days 9, 16 and 22 across all treatments were either similar to or higher than CON (Table 3.4).

Biofilm PP on Day 8 in 1X was significantly higher ($p = 0.01$) but significantly lower in 1000X ($p = 0.03$) compared to CON (Figure 3.7). 10X and 500X were not different from CON for the same time period. On Day 21, rates in 10X, 500X, and 1000X were significantly lower (ANOVA, $p < 0.05$) than CON while, in 1X, no difference was observed.

On Day 8, biofilm Chl *a* in 1X was similar to CON while in 10X and 500X, it was significantly lower ($p < 0.05$) (Table 3.5). In contrast, Chl *a* in 1000X was significantly higher

than the CON ($p < 0.05$). By Day 21, however, algal biomass was significantly lower than CON in 10X, 500X, and 1000X ($p < 0.05$) while no difference was noted for 1X.

Although BP in 1X, 10X, and 500X on Day 8 was not significantly different from CON, BP in 1000X was significantly lower ($p = 0.03$) than CON (Table 3.5). On Day 21, rates in 1X and CON were similar while, those in 10X, 500X, and 1000X were significantly lower ($p < 0.05$) than CON.

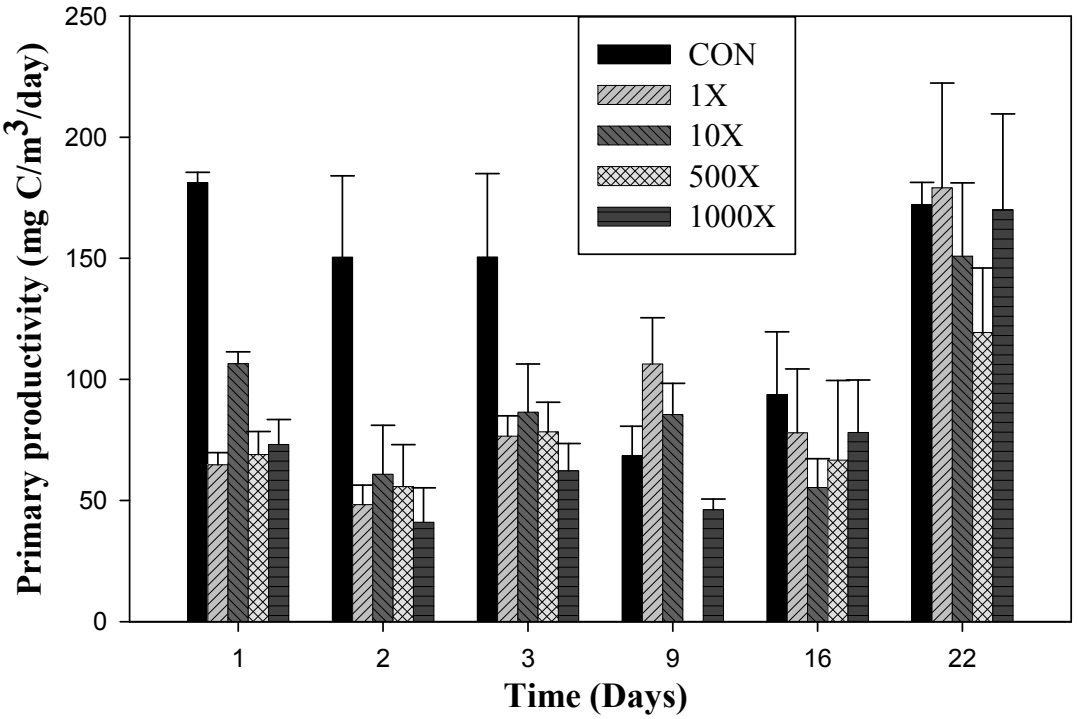


Figure 3.5 Pelagic primary productivity (PP) rates in CON (control), 1X, 10X, 500X and 1000X (mixture of 8 herbicides at 1, 10, 500 and 1000 times environmentally relevant concentrations, respectively), mesocosms. Error bars represent standard deviation, n = 4.

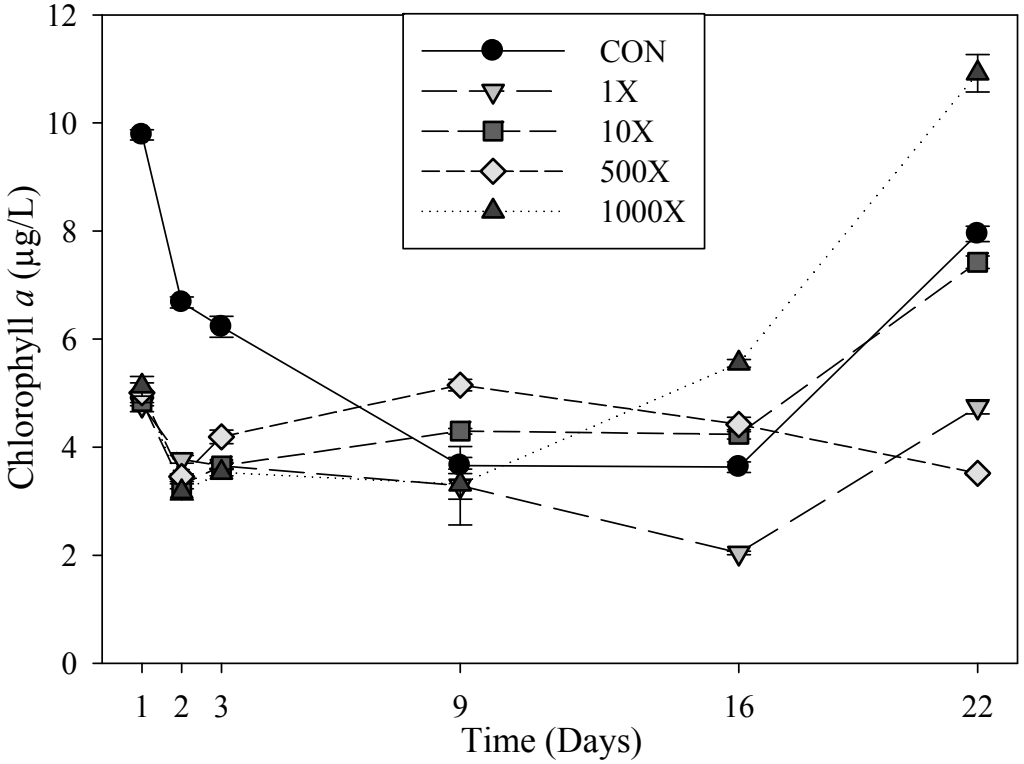


Figure 3.6 Pelagic chlorophyll *a* (Chl *a*) content in CON (control), 1X, 10X, 500X, and 1000X (mixture of 8 herbicides at 1, 10, 500 and 1000 times environmentally relevant concentrations) mesocosms. Error bars represent standard deviation, n = 4.

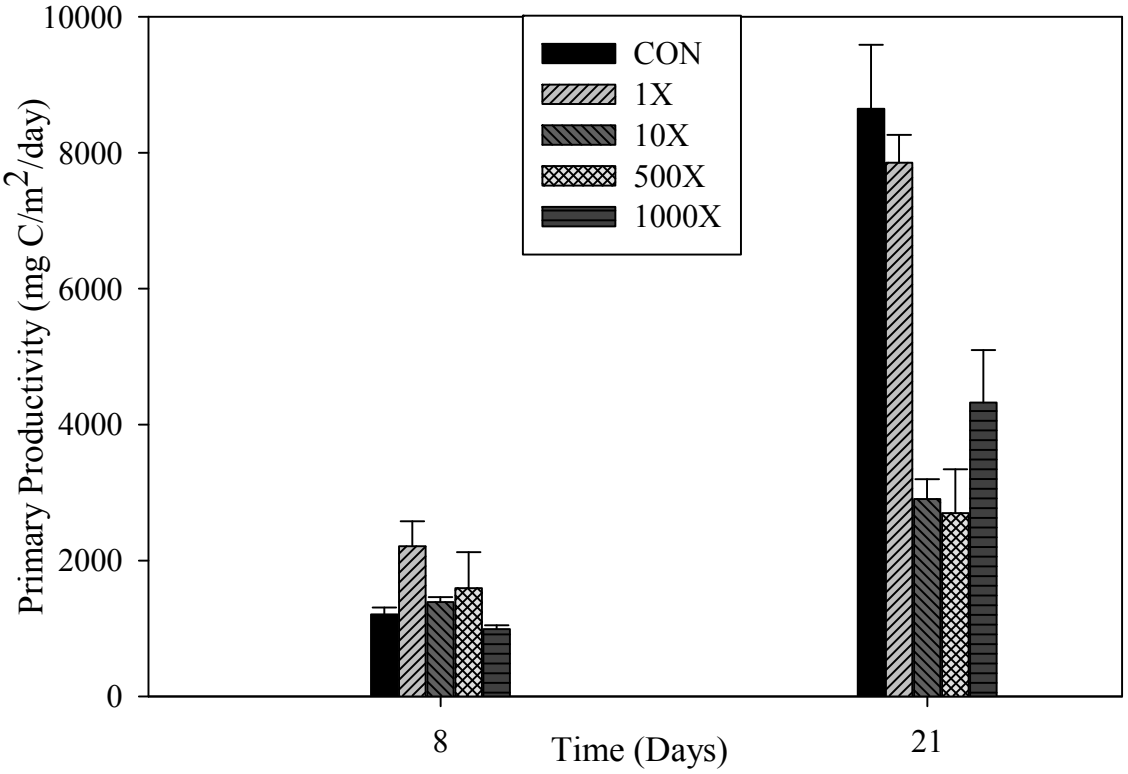


Figure 3.7 Biofilm primary productivity (PP) in CON (control), 1X, 10X, 500X, and 1000X (mixture of 8 herbicides at 1, 10, 500 and 1000 times environmentally relevant concentrations) mesocosms. Error bars represent standard deviation, n = 4.

3.4 Discussion

3.4.1 Glyphosate Treatment (GLY)

The results from this study indicate that glyphosate at 225 μg/L (GLY) can have a profound effect on the productivity of aquatic microbial communities over relatively short time periods. This concentration was about 18 times higher than the published lowest NOEC (12.6 μg/L) and 22.5 times higher than lowest EC_{50} (10 μg/L) [Pesticide Action Network Pesticide Database, http://www.pesticideinfo.org (accessed 10 June 2010)]. As expected by this high concentration and its herbicidal activity, glyphosate reduced PP rates and Chl *a* content in pelagic mesocosm communities below CON values. Results presented here are similar to those observed by other researchers (Peterson et al., 1994; Vera et al., 2010; Wong, 2000). Vera and co-workers (2010), for example, treated artificial outdoor mesocosms with glyphosate at 8000 μg/L and observed a 30 – 33 % reduction in algal abundance and 30 – 60 % reduction in PP below control levels. Glyphosate interferes with shikimic acid pathway disrupting synthesis of aromatic amino acids. The primary target of glyphosate, 5-enolpyruvyl shikimate 3-phosphate synthetase (EPSP ase), catalyzes the formation of 5-enolpyruvyl shikimate 2-phosphate (EPSP), an intermediate in the shikimic acid pathway (Amrhein et al., 1980; Boocock and Coggins, 1983; Hollander-Czytko and Amrhein, 1987). In the present study, the presence of glyphosate at 225 μg/L reduced Chl *a* and PP immediately following treatment. Although initially depressed, algal biomass was greater than CON by Day 21. Results presented here differ somewhat from other researchers (Peterson et al., 1994; Wong, 2000). Wong (2000), for example, investigated effects of glyphosate on a green alga, *Scenedesmus quadricauda*, in a laboratory experiment. He observed that algal growth, photosynthetic rate and Chl *a* were not affected by 200 μg/L glyphosate while, at 2000 μg/L, growth was significantly inhibited, photosynthetic rate was reduced to 60% of control and

Chl *a* content was significantly reduced. At 20000 μg/L or more, glyphosate completely inhibited algal growth and Chl *a*, and reduced photosynthetic rates below detection. In another laboratory study, Peterson et al. (1994) investigated the effects of glyphosate on 11 algal species and determined that 2848 μg/L glyphosate inhibited $NaH^{14}CO_3$ uptake by 73-77 % in two algal species, while in another two species, only 3-18 % inhibition was noted. As well, glyphosate was found to be very toxic to diatoms and nitrogen-fixing cyanobacteria. Results obtained in the current wetland mesocosm study confirm the general observation that glyphosate does affect algal biomass and productivity. More importantly, however, the wetland mesocosm results indicate these effects occurred at glyphosate concentrations lower than those utilized in the other laboratory studies discussed earlier. Furthermore, the outcome of the mesocosm study underlines the importance of using higher tiered multi-community effects studies to estimate pesticide risk to aquatic communities.

Another interesting observation from the mesocosm study was that the pelagic algal community was slow to recover after glyphosate exposure. Although PP and Chl *a* had increased significantly by Day 22 compared to Day 1, they were still lower than CON. The slow recovery may be linked to glyphosate dissipation and how it partitions into sediments. The dissipation half-life of glyphosate in the mesocosm water was about 9.2 to 11.4 days (Sura et al., unpublished data), longer than 4.2 days observed in outdoor mesocosms by Vera et al. (2010). Glyphosate, which partitions into the sediments, however, has a longer dissipation half-life in sediment and slowly desorbs in response to the faster dissipation in water column (Barja and dos Santos Afonso, 2005). Glyphosate is transformed into aminomethyl phosponic acid (AMPA) both in water and sediments and subsequently broken down into CO_2, NH_4^+, and inorganic phosphate (Franz et al., 1997). AMPA is usually detected more frequently than its parent

compound and its dissipation rate is slower than that of glyphosate (Kolpin et al., 2006). Desorption of glyphosate from sediments means longer apparent persistence than would be indicated by its dissipation half-life in water. But AMPA is known to have little toxicity to algae (Giesy et al., 2000). Consequently, the extended period of glyphosate presence in the water column as a result of slow release of sorbed glyphosate from sediment, may slow the process of recovery for microbial communities.

Because the shikimic acid pathway is present in both bacteria and plants (Amrhein et al., 1980; Boocock and Coggins, 1983; Steinrucken and Amrhein, 1984), there was an expectation that glyphosate might affect mesocosm bacterial communities in a similar fashion to phytoplankton communities. Pelagic BP and bacterial numbers, however, were not affected by glyphosate at 225 μg/L. These results suggest that the threshold concentration at which bacterial communities are inhibited may be higher than that for algae. Another explanation, however, may be functional redundancy within wetland bacterial communities. Glyphosate usage has continuously increased in the Prairie region and indeed across Canada and worldwide since its first introduction in 1974 (Franz et al., 1997). Microbial communities exposed to herbicides for relatively long periods tend to develop permanent changes in their community structure (Dorigo et al., 2004). These changes occur as a result of replacement of sensitive species with those that are either resistant to the herbicide (Gonod et al., 2006) or those capable of herbicide mineralization through enzyme induction or genetic adaptation (Alexander, 1999; Lancaster et al., 2009). Bacterial communities capable of utilizing organophosphonates, such as glyphosate, as a source of phosphorus have been shown to occur in various environments. For example, Schowanek and Verstraete (1990) screened various environmental matrices such as soil, water, activated sludge, and waste treatment effluents for presence of glyphosate-utilizing

microorganisms. They found that microorganisms capable of breaking the carbon-phosphorus bond and using glyphosate as a source of phosphorus are widespread in the environment. Balthazor and Hallas (1986) also isolated similar glyphosate-utilizing microorganisms from activated sludge. The replacement of sensitive species with those resistant to herbicides or capable of degradation means that there is little disturbance to overall ecosystem processes like BP (Lawton and Brown, 1993) and ecosystem integrity is preserved (ecosystem redundancy). The presence of glyphosate-utilizing microorganisms or those resistant to it (Pesce et al., 2009) may explain the lack of negative effect of glyphosate on bacterial communities observed in the present study.

Exopolysaccharide (EPS) produced by biofilm bacteria (Amellal et al., 1998) provides a certain degree of protection for biofilm communities from various environmental stresses such as UV radiation, pH changes, osmosis and dehydration as well as chemical biocides and antimicrobial agents (Costerton et al., 1999; Flemming, 1993). Small molecules, such as glyphosate, however, may be able to penetrate EPS. Furthermore, once biofilm algae are exposed to this herbicide, negative effects may ensue such as the decreased algal productivity and biomass observed in this study. BP was also negatively affected by glyphosate and this observation may have something to do with the fact that biofilms have microbial communities that are physiologically interdependent and metabolically cooperative for their existence. In the mesocosm study, excreted organic carbon from primary producers on which the biofilm bacteria depend, may have decreased coincident with decreased algal productivity and biomass. This decrease may have in turn contributed to the decrease in biofilm BP observed. As well, bacterial communities dependent on labile carbon excreted by biofilm algae, were likely not adapted to using anthropogenic substances, such as glyphosate, as a carbon source.

3.4.2 Auxin-Type Herbicide Mixture Treatment (AUX)

When herbicides are present in the environment as mixtures, they may exert an effect in four possible ways, including: i) simple concentration addition, ii) a more complex synergism, iii) a decreased activity (antagonism) (Thompson, 1996) and iv) no change in toxicity (independent action). According to the concept of *'concentration addition'*, a simple addition of concentrations of different components is reasonable for a mixture of chemicals with similar modes of action. The total effect of the mixture, therefore, can be reasonably predicted based on additive toxicity (Faust et al., 2001). Because all six herbicides in AUX had similar modes of action (mimicking those of plant auxins) their total concentration can be calculated by adding concentrations of each herbicide giving a total in AUX of 220 μg/L. Although data on mixture toxicity of auxin-type herbicides could not be found, information on 2,4-D toxicity is available (Boyle, 1980; Kobraei and White, 1996; Wong 2000 – discussed below) and can be useful in discussing effects of the AUX treatment.

Auxin-type herbicides behave similarly to naturally occurring auxins and also induce synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase via increased expression of specific ACC synthase genes (Abel and Theologis, 1996; Kende and Zeevaart, 1997; Wei et al., 2000). ACC synthase is an important enzyme in the ethylene biosynthesis pathway and catalyses the conversion of *S*-adenosylmethionine to ACC which, in turn, forms ethylene. Ethylene is a plant hormone responsible for plant growth regulation, epinasty, senescence, and stimulation of abscisic acid (ABA) production (Kende and Zeevaart, 1997). Auxins, ethylene, and ABA acting together have been shown to bring about the death of plant tissue (Grossmann, 1998, 2000). According to the literature, auxin-type herbicides exhibit opposing effects on plants at different concentrations. At low concentrations, growth by cell division and elongation is usually

stimulated and at higher concentrations, various growth abnormalities are induced eventually leading to the death of the plant tissue (Grossmann, 1998, 2000). Such stimulatory effects on the growth of the living organisms by toxic substances at low and sublethal concentrations, is similar to the commonly observed phenomenon, hormesis (Stebbing, 1982). In AUX, negative effects were observed immediately following treatment, while stimulatory effects were seen on Days 9 and 22 because, based on half-lives of the six auxin-type herbicides (Sura et al., unpublished data), the total concentration of auxin-type herbicides would have decreased.

Although PP and Chl *a* in AUX declined immediately following treatment on Days 1, 2, and 3, by Days 9 and 22 both variables were significantly higher than those on Day 1 (indicating recovery) and also higher than those in CON (indicating the stimulus effect of the auxin-type herbicides) (Figure 3.4A and B). Results presented here are similar to those found in the literature. For example, 2,4-D stimulated algal growth and production at 200 μg/L (Wong, 2000) and at 2000 μg/L (Boyle, 1980; Kobraei and White, 1996). Thus at concentrations below 2000 μg/L, these auxin-type herbicides have been shown to promote phytoplankton growth. In the present study, although the phytoplankton community was initially inhibited in AUX, it recovered quickly and by the end of the study, PP and Chl *a* were significantly higher than CON. The 220 μg/L mixture of auxin-type herbicides used in this study was in the concentration range where stimulation of algal growth has been reported in the literature as discussed above. And stimulation was likely a direct result of concentration addition of similarly acting herbicides. Reported $NOEC_{eco}$ for 2,4-D was 10 μg/L on macrophytes (vegetative growth) (Forsyth et al., 1997) while reported $LOEC_{eco}$ values range from 100 μg/L on macrophytes [vegetative growth; (Forsyth et al., 1997)], ≤ 500 μg/L [algal growth and production; (Boyle, 1980)] to ≤ 2000 μg/L [algal growth and production; (Kobraei and White, 1996)]. Although concentrations of each

auxin-type herbicide in AUX was below $LOEC_{eco}$ for 2,4-D (100 μg/L), the total concentration of auxin-type herbicides exceeded the $LOEC_{eco}$. It is therefore likely that herbicide effects noted in AUX were due to their additive effect. Faust et al. (2001) demonstrated that low concentrations of triazines, that individually did not cause statistically significant responses, contributed to the overall negative effects when applied as a mixture. In the mesocosm study here, the most likely explanation for the observed results was that the combined concentrations of auxin-type herbicides stimulated rather than suppressed phytoplankton growth.

Although biofilm PP Chl *a*, and BP in the AUX mesocosm were initially stimulated (similar to the pelagic communities), stimulation was short lived. By experiment end PP and Chl *a* were significantly lower than CON, while BP did not differ from CON. The reason for the decline in PP and Chl *a* relative to the control is not clear. It is known, however, that herbicides partition into biofilms and are metabolized by the microbial community (Lawrence et al., 2001). It may be that bacterial degradation of herbicides within the biofilm produced metabolites that were more toxic than the parent compound. Further study is required.

3.4.3 1X, 10X, 500X, and 1000X Treatments

Suppression of pelagic PP and Chl *a* among these four treatments lasted for differing periods of time. In 1X and 10X treatments, for example, inhibition of PP and Chl *a* was brief only lasting for the first three days. The short inhibition time observed in these two latter treatments may be due to reduced herbicide concentrations as a result of photolytic breakdown and/or microbial degradation. Although the 1X treatment inhibited PP and Chl *a* for only the first three days, it is important to consider that these concentrations reflect those frequently detected in water bodies across the prairies. Similarly, in 10X total herbicide mixture concentration was still below the Water Quality Guidelines for Protection of Aquatic Life. The presence of residual concentrations

of these herbicides and other pesticides in prairie aquatic ecosystems as noted by Donald et al. (2007), could theoretically inhibit primary productivity for longer than the 1-week period observed in this study.

Inhibition of PP and Chl *a* in the 500X and 1000X treatments lasted longer than 1 week. This longer inhibition period is likely related not only to higher overall herbicide concentrations, but longer dissipation rates for some herbicides. Among herbicides other than glyphosate, bromoxynil was short lived ($T_{1/2}$ = 5 to 8 days) while mecoprop and clopyralid had longer half-lives ($T_{1/2}$ = 14 to 44 days). Dichlorprop and 2,4-D concentrations (both applied as esters), steadily decreased likely due to slow hydrolysis (Sura et al., unpublished data). The dissipation half-life of glyphosate ranged from 6 to 10 days. Results from the AUX treatment indicated that 1000X ERC of each auxin-type herbicide stimulated PP and Chl *a*, contrary to what was observed here in the 1000X (auxin-type herbicides plus glyphosate and bromoxynil). Based on observations from the GLY treatment and the fact that bromoxynil is a photosynthetic inhibitor (Tomaso, 1994), negative effects in the 500X and 1000X may therefore be due to glyphosate alone or glyphosate and bromoxynil. Peterson et al., (1994), however, observed that bromoxynil at 280 μg/L did not inhibit algal growth. In another study bromoxynil did not adversely affect 17 strains of green algae at concentrations as high as 10 mg/L (Cullimore, 1975). Based on this information, bromoxynil, at the concentration used (11.3 μg/L), likely did not have any effect on PP and Chl *a*. Negative effects observed, therefore, were likely due to glyphosate. Given the half-life of glyphosate (6 to 10 days) it is not surprising that recovery was observed on Day 22 in both 500X and 1000X. Based on observations in this study and evidence from the literature, glyphosate in 1000X appears to be the herbicide inhibiting PP as well as masking the stimulatory effects of the auxin-type herbicides.

Bromoxynil and all auxin-type herbicide concentrations used to treat mesocosms were below the established NOEC and EC_{50} values. Glyphosate concentration was below NOEC (12.6 μg/L) and EC_{50} (10 μg/L) [Pesticide Action Network Pesticide Database, http://www.pesticideinfo.org (accessed 10 June 2010)] only in 1X and 10X. Even though concentrations of individual herbicides do not exceed water quality standards, they may pose a threat to the ecosystem when present as a mixture. It is important to consider the presence of other similarly or dissimilarly acting chemicals in the ecosystem before applying an $NOEC_{eco}$ or $LOEC_{eco}$. One approach for resolving this issue would be to establish the 'ecosystem safe chemical burden/load', i.e., the total concentration of all plausible bioactive chemicals in the environment that is safe for the most sensitive endpoint in the ecosystem. Thus, there is a need to reassess currently established $NOEC_{eco}$, $LOEC_{eco}$, and other guidelines by considering all relevant bioactive chemicals and their interactions in the ecosystem. The concept of developing a safe chemical burden for ecosystems may seem tedious in the presence of the large number of chemicals and undefined number of interactions in the environment, but, ultimately, it may be one of the best ways to handle mixtures of bioactive chemicals in the environment.

While biofilm PP, Chl *a*, and BP in 1X were similar to those in CON on both sampling days, all were suppressed below CON in 10X, 500X and 1000X on Day 21. Negative effects occurring later in experiments have been observed in other studies (Pesce et al., 2006). Natural riverine microorganisms exposed to the herbicide, diuron, for 21 days, for example, only showed effects after the first 6 days (Pesce et al., 2006). There are a number of explanations for the decline in PP and Chl *a* observed at end of the experiment. As noted above, herbicide adsorption by the biofilm with subsequent degradation to more toxic metabolites may have occurred. Alternately, the declines observed may be due to trophic interactions as a result of herbicide exposure. It is

known for example, that invertebrates prey on protozoans (Pace and Funke, 1991; Stoecker and Capuzzo, 1990). If the invertebrate population declined due to herbicide toxicity, protozoan numbers could increase. Increasing protozoan grazing in turn could reduce the biofilm volume (Mohamed et al., 1998). While such a mechanism cannot be verified in the mesocosm study here it remains a possibility deserving of further investigation.

Finally, another explanation for delayed negative effects might be slow recovery. In one study, biofilms took approximately 5 weeks to recover after removal of the pesticide, diuron, from the surrounding medium (Dorigo et al., 2010b). Restoration and subsequent recovery took place by recruitment of new microbial community members. Recruiting new members to an early stage biofilm has been shown to be easier than to a mature biofilm (Iserentant and Blancke, 1986). In light of the above evidence and the fact that the duration of the present study was only 3 weeks, it may be that biofilms in the 10X, 500X, and 1000X may be in the slow process of recovery or under grazing pressure or a combination of both.

In summary, the present study provided evidence that herbicides even at low concentrations (that is, below Water Quality Guidelines for the Protection of Aquatic Life) exhibit effects on wetland microbial communities when present as mixtures. Negative effects were short term in 1X and 10X treatments and the microbial communities showed signs of recovery by Day 21. Prolonged negative effects as a result of slow recovery, however, were observed in GLY, AUX, 500X and 1000X. In this study, effects were studied after a single application of herbicide mixture. Considering that most herbicides on the prairies are applied once each spring, results presented here are perhaps more realistic than if herbicides had been applied as a repeated (press) application.

This study also demonstrated that a mixture of auxin-type herbicides at concentrations well below water quality guideline levels and at relevant environmental concentrations can exert effects, and that effects are more likely due to concentration addition. Based on the results presented here, exposure to herbicide residue mixtures at low concentrations for prolonged periods (chronic exposure) may permanently change wetland microbial communities. Instead of using current $NOEC_{eco}$, $LOEC_{eco}$, and other guideline values to assess risk to aquatic ecosystems, there is a need to establish new guidelines that take into consideration the environmentally relevant pesticide mixtures and their interactions in the ecosystem. Based on the evidence presented in this study, glyphosate has a higher potential than mixtures of auxin-type herbicides to inhibit PP and Chl *a* in wetland ecosystems.

3.5 Further Research

The following chapter (CHAPTER 4) is prepared in the form of a manuscript and will be submitted for publication in a peer-reviewed journal. This chapter discusses the enclosure study where effects of a herbicide mixture on microbial communities in four ponds with varying salinities were investigated. The herbicide mixture concentration used in this study represents a maximum-exposure scenario where the concentrations were equivalent to those applied to crop lands. These herbicide concentrations were higher than those used in 1000X treatment in mesocosm study (CHAPTER 3) for seven of eight herbicides while lower for glyphosate. This study also utilizes the *in situ* enclosures to understand effects on microbial communities.

4. EFFECT OF A HERBICIDE MIXTURE ON MICROBIAL COMMUNITIES IN FOUR PONDS WITH VARYING SALINITIES IN A PRAIRIE WETLAND ECOSYSTEM: AN ENCLOSURE APPROACH

Abstract

The wetlands in the Prairie pothole region of Saskatchewan and Manitoba serve an important role in providing wildlife habitat, water storage and water filtration. Prairie wetlands display a wide range of water quality parameters such as salinity and nutrients with sulfate as the dominant ion for the most saline ponds. The differences in salinity and nutrients are reflected in the composition of plant communities and their productivity. These wetlands are regularly interspersed among intensive agricultural operations where pesticides are commonly used. Mixtures of herbicides are often detected in prairie waters including rivers, drinking water reservoirs, and wetlands. Four wetlands, one freshwater (Pond 109) and three saline ponds (Ponds 02, 50, and 67) in the St. Denis National Wildlife Area, Saskatchewan, Canada, were selected to study the effects of a mixture of eight herbicides (2,4-D, MCPA, dicamba, clopyralid, bromoxynil, mecoprop, dichloroprop, and glyphosate at maximum-exposure concentrations), on wetland microbial communities using a outdoor enclosure approach. Six enclosures (three controls and three treatments) were installed in each pond and primary productivity, algal biomass and bacterial productivity were measured in both pelagic and biofilm communities over a period of 28 days. The herbicide mixture had a stimulatory effect on primary productivity in the nutrient-sufficient freshwater pond while no stimulatory effect was observed in the nutrient-deficient saline ponds. The differences observed in the effects of the herbicide mixture appear to be related to the nutrient status and the salinity of these ponds.

4.1 Introduction

The Prairie pothole region (PPR) across south central Canada and the north central United Stated contains more than four million wetlands which collectively cover 15 % to 25 % of the region (Mitsch and Gosselink, 2000). The total wetland area in North America has declined substantially, mostly due to human activities such as drainage (Dahl, 2000). Prairie wetlands serve an important role in providing water storage, water filtration and wildlife habitat. Prairie wetland food-webs consist of primary producers (free-living and attached algae, submerged and emergent plants), bacteria, benthic invertebrates, emergent insects and higher trophic level mammals and migrating waterfowl. These aquatic ecosystems are key ecological features of the prairie region, supporting 50-80 % of the North American waterfowl population and 50 % of other migratory birds each year (Batt et al., 1989).

The glaciated plains of the North American continent have unique hydrological and hydrogeological characteristics due to the combination of the semiarid, cold climate and the glacial deposits that underlay the area. The glacial deposits are a rich source of mineral nutrients and the closed nature of wetland basins means that nutrients are trapped and recycled instead of being flushed out by surface runoff. Prairie wetlands located in lower landscape elevations are generally more saline than those located in the higher parts (LaBaugh, 1989; Sloan, 1972). Wetlands and lakes of the northern prairie region have sulphate salts originating from glacial deposits (van der Valk, 1989). At the St. Denis National Wildlife Area, Saskatchewan, Canada (study site), specific conductivities of pond water range from less than 400 μS/cm, for shallow marshes, to over 24000 μS/cm for terminal wetlands, with sulphate as the dominant anion for the more saline ponds (Driver and Peden, 1977). The difference in salinity is reflected in the composition of plant communities (Stewart and Kantrud, 1972).

One of the most important features of the PPR is that wetlands are interspersed among agricultural fields where pesticides are commonly used (Donald et al., 1999; Waiser and Robarts, 1997). These pesticides eventually reach surrounding wetlands via spray drift, aerial deposition, surface runoff, or ground water flow (Grover et al., 1988; Waite et al., 1992). As a result, they are frequently detected in prairie wetlands across this region (Donald et al., 1999, 2001, 2007; Waite et al., 2004). The seven herbicides most commonly found in prairie waters (drinking water reservoirs, wetlands and farm dugouts) are: 2,4-D [2-(2,4-dichlorophenoxy)acetic acid], MCPA [2-(4-chloro-2-methylphenoxy)acetic acid], dicamba [3,6-dichloro-2-methoxybenzoic acid], clopyralid [3,6-dichloropyridine-2-carboxylic acid], dichlorprop [2-(2,4-dichlorophenoxy)propanoic acid], mecoprop [2-(4-chloro-2-methylphenoxy)propanoic acid], and bromoxynil [3,5-dibromo-4-hydroxybenzonitrile] (Donald et al., 2007). These herbicides are also among the most widely used in PPR for crop production (Waiser and Holm, 2005). There is, however, limited information regarding the environmental fate (Degenhardt et al., 2011) and toxic effects of these herbicide mixtures in prairie wetlands, and how their fates and impacts vary with wetland hydrology. In fact, DeLorenzo et al. (2001) opined that the influence of water quality on pesticide toxicity to microorganisms needed to be researched. Water quality parameters such as pH, salinity and nutrient concentrations may influence the effect of pesticides on microbial communities. Consequently, the results presented here, comparing microbial community responses to herbicide mixtures under nutrient-enriched freshwater (Pond 109) and lower nutrient saline (Ponds 02, 50 and 67) conditions, would be useful contributions to the existing literature (DeLorenzo et al., 2001).

In this study, a multi-trophic outdoor enclosure system was used to mimic the wetland ecosystem and to investigate effects of a herbicide mixture (MCPA, clopyralid, dicamba,

dichlorprop, mecoprop, 2,4-D, bromoxynil, glyphosate) on wetland microbial communities (algae and bacteria) varying in salinities and nutrient concentrations. The intent of this study was to provide much needed knowledge regarding effects of this environmentally relevant herbicide mixture on prairie wetland microbial communities.

4.2 Materials and Methods

4.2.1 Study Site and Design

Four wetlands, pond 109 (P109), pond 2 (P02), pond 50 (P50), and pond 67 (P67) were selected based on their salinities in the St. Denis National Wildlife Area (52°02' N 106°06' W), located 45 km east of Saskatoon, SK, in the Prairie Pothole Region (PPR) of Saskatchewan. P109 is a freshwater wetland (~500 µS/cm) while P02 (~3597 µS/cm), P50 (~3875 µS/cm), and P67 (~4535 µS/cm) are saline in the order of their increasing specific conductivity. Six enclosures were installed in each wetland in early May 2008 and were allowed to acclimatize *in situ* for 4-6 weeks before the start of the experiment (Figure 4.1 and Figure 4.2). Each enclosure is a circular plastic hollow tube, measuring 100 cm (diameter) x 160 cm (height) and open on both ends. These enclosures were pushed into the bottom sediments of each pond, isolating part of the pond water and sediments. To ensure no leakage, approximately 25 to 30 cm of the enclosure bottom was pushed into the sediments. Six enclosures in each pond were placed randomly in close proximity to each other for easy sampling. Heights of the enclosures were adjusted by cutting off the excess so that only 35-40 cm was above the water surface level. Water depths were measured and volumes were calculated for all enclosures one day prior to treatment. Water volumes ranged from approximately 560 L to 750 L.

4.2.2 Herbicide Treatment

A mixture of eight commercially formulated herbicides was used (Table 4.1) with the target concentration for each being the expected environmental concentration (EEC). EEC is the

calculated concentration of a herbicide, in various environmental compartments, for example, water, based on maximum exposure scenarios. In this study, EEC was based on 100 % direct overspray of herbicide at recommended field application rate on a 0.5-m deep water body (Cessna et al., 2006). For example, glyphosate, at the recommended application rate of 360 g/ha, when applied to a 0.5-m deep pond, will result in a water concentration of 72 μg/L. Concentrations calculated in this manner for each herbicide became the target concentration. These concentrations are approximately 10 times (for Dicamba) to 50 times (for MCPA) greater than their respective herbicide Canadian water quality guidelines for the protection of aquatic life in freshwater (Table 4.1). Three enclosures in each pond were randomly assigned as treatments and received the herbicide mixture while the other three served as controls. The same proportional herbicide mixture was added to each treatment enclosure in all four ponds. The three treatment enclosures in each pond could represent pseudoreplication; however, it is practically impossible to find two or more ponds with similar biotic and abiotic characteristics.

Figure 4.1 Photograph showing six enclosures installed in May 2008 in Pond 109 at St. Denis National Wildlife Area, Saskatchewan, Canada.

Figure 4.2 Photograph showing six enclosures installed in May 2008 in Pond 02 at St. Denis National Wildlife Area, Saskatchewan, Canada.

Table 4.1. List of herbicides used, their trade names, active ingredients, concentrations, recommended application rates, and enclosure fortification target concentrations in Ponds 109, 02, 50 and 67 at St. Denis National Wildlife Area, Saskatchewan, Canada.

Herbicide	**Trade name**	**Herbicide formulation**	**Concentration of active ingredient (acid equivalent) (g/L)**	**Recommended application rates for cropland (g/ha)[a]**	**Target concentration in treated enclosures (active ingredient) (µg/L)**	**Guideline value[b] (µg/L)**
2,4-D	Nufarm Estaprop PLUS	2-ethylhexyl ester	282	495	100	4
MCPA	Nufarm MCPA Amine 500	Dimethylamine salt	500	625	127	2.6
Clopyralid	Lontrel 360	Monoethanolamine salt	360	266	53	NA[c]
Dicamba	Oracle	Dimethylamine salt	480	468	95	10
Bromoxynil	Pardner	Octanoate / heptanoate ester	280	308	62	5
Dichlorprop	Nufarm Estaprop PLUS	2-ethylhexyl ester	300	525	106	4
Mecoprop	Mecoprop	Potassium salt	150	895	181	4
Glyphosate	Glyphos	Isopropylamine salt	360	360	72	65

[a] These application rates represent recommended maximum safe rates for wheat and barley crops in Saskatchewan and Manitoba (Saskatchewan Ministry of Agriculture, 2008).
[b] Water Quality Guideline for the Protection of Aquatic Life in Freshwater (CCME, 1999).
[c] NA = Not available.

4.2.3 Sampling and Water Analysis

Surface water temperature, pH, dissolved oxygen (DO), alkalinity and specific conductivity were measured between 10:00 and 11:00 h on each sampling date using a YSI 650MDS data display and logging unit connected to a 600XLM-0 multi-parameter water quality monitoring probe (YSI Inc., Ohio, USA). Every two weeks, composite water samples were collected from each enclosure as well as four ponds (outside the enclosures) for nutrient analyses including total phosphorus (TP), total dissolved phosphorus (TDP), ammonium nitrogen (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), dissolved organic carbon (DOC), and particulate carbon and nitrogen (PC, PN) and major ions including sulfate (SO_4^{-2}) and magnesium (Mg^{+2}). Samples were prepared and filtered according to established methods (EC, 1992), placed on ice, and transported to the laboratory for analysis. NH_4^+ and TP were analyzed using a Seal Colorimeter AA-3 (Seal Analytical, Norderstedt, Germany). DOC was analyzed using DOC analyzer (Tekmar-Dohrmann Phoenix 8000, Ohio, USA). Calcium (Ca), magnesium (Mg), sodium (Na), and potassium (K) were also measured using ion chromatography (ICS-1000, Dionex Canada Ltd., ON, Canada) (EC, 1992). Particulate phosphorus (PaPh) was calculated as difference between dissolved and total phosphorus (PaPh = TP – TDP). Sestonic ratios (PN:PaPh), (PaPh:PC), and (PN:PC) were also calculated. Nutrient status was assessed based on the sestonic ratios according to (Healey and Hendzel, 1980).

For pelagic community sampling, water samples from each enclosure of all four ponds were collected on Days -3 (pre-treatment), 1, 2, 3, 7, 14, 21, and 28 (post-treatment) into clean 2-L amber PVC bottles. This water was subsequently screened through a 150-µm Nitex mesh screen (Dynamic Aqua-Supply Ltd., BC, Canada) to remove large zooplankton. The screened water was

subsequently used to determine primary productivity (PP), chlorophyll *a* (Chl *a*), bacterial productivity (BP).

For the biofilm community sampling, attached communities (biofilms) were grown *in situ* on 2.54-cm^2 diameter glass coverslips (Figure 4.3). Prior to deployment, coverslips were loaded onto PVC plates, previously etched with tracks to hold the round coverslips. The plates were then fitted onto a floating plastic platform. Platforms were deployed into each enclosure of all four ponds one day prior to treatment. Four float balls mounted on four corners of the platform kept coverslips at a constant depth of approximately 20 cm below water surface. Coverslips with attached biofilms were harvested after 8, 15, and 21 days of growth, post-treatment. These biofilms were used to measure PP, BP, Chl *a* content, and carbon source utilization patterns (BIOLOG technique).

Figure 4.3 Photograph showing floating plastic platform fitted with PVC plates holding coverslips deployed at a water depth of approximately 20 cm in each enclosure.

4.2.4 Pelagic Community Analysis

4.2.4.1 Measurement of primary productivity (PP)

PP was determined using a standard light/dark bottle ^{14}C method which measures the ability of phytoplankton to take up and incorporate tracer amounts of radioactive isotopes into organic matter during photosynthesis (Wetzel and Likens, 1991). Volumetric rates of PP (mg C m^{-3} h^{-1}) were calculated from ^{14}C incorporation rates and ^{12}C concentrations (from temperature, pH and alkalinity data) available to phytoplankton (Robarts et al., 1992). Daily rates were estimated by multiplying hourly rates by 10 (Cole et al., 1988).

Alkalinity of water was determined by end point titration with 0.01N H_2SO_4 solution (Clesceri et al., 1998) using a TitraLab TIM850 titration system with SAC80 autosampler (Radiometer Analytical SAS, France) linked to TitraMaster 85 software.

4.2.4.2 Measurement of bacterial productivity (BP)

BP was determined by rate of incorporation of a radioactively-labelled nucleotide (^{3}H-thymidine) into nucleic acids (Robarts and Wicks, 1989). BP rates were calculated from ^{3}H-thymidine incorporation using a conversion factor for a eutrophic lake of 2.0 x 10^{18} bacterial cells produced per mole ^{3}H-thymidine (Bell et al., 1983; Coveney and Wetzel, 1988). A factor of 20 fg C per bacterial cell was then used to convert cell numbers to an estimate of carbon produced (Lee and Fuhrman, 1987; Reitner et al., 1999). Because the carbon content of most bacterial cells is in the 10 to 20 fg C /cell range (Cotner and Biddanda, 2002), the upper limit was chosen to represent carbon content of bacterial cells in eutrophic ecosystems. Daily volumetric rates were estimated by multiplying hourly rates by 24 (Cole et al., 1988).

4.2.4.3 Estimation of phytoplankton biomass

Phytoplanktonic biomass was estimated as Chl *a* (Wetzel and Likens, 1991). Water samples were filtered through 47-mm Whatman GF/C filters (nominal pore size 1.2-µm). Chl *a* was extracted using a boiling ethanol technique and subsequently analysed fluorometrically using a Turner Design Model 10-AU digital fluorometer (Turner Designs, Sunnyvale, CA) (Waiser and Robarts, 1997, 2004).

4.2.4.4 Estimation of protein and carbohydrate content

Water samples were filtered through 47 mm GF/C filters (Whatman; nominal pore size 1.2 µm) for protein and carbohydrate analysis. Particulate protein was measured using the Folin-Lowry method (Rausch, 1981). Absorbance was measured using a UV-Visible Spectrophotometer (UV-160 1PC, Shimadzu, Japan) at 750 nm. Particulate carbohydrate was

measured by the phenol-sulfuric acid method (Pick, 1987). Absorbance was measured using the UV-Visible Spectrophotometer at 485 nm.

Sestonic protein-to-carbohydrate ratios (PRO:CHO) were used to interpret nutrient status in the enclosures. Ratios > 1.2 indicated no nutrient deficiency, while those <0.7 indicated severe nutrient deficiency (Pick, 1987).

4.2.5 Biofilm Community Analysis

4.2.5.1 Measurement of primary productivity (PP)

For biofilm PP, coverslips were randomly harvested and placed in crystallization dishes containing 20 mL of 0.2-μm filter sterilized water and 450 μL $NaH^{14}CO_3$. Dark control dishes were covered with foil and all were incubated for one hour. Coverslips were then removed, individually placed in 50 mL Falcon tubes containing 50 mL 0.2-μm filter sterilized water, placed on ice and transported to the laboratory (Waiser, 2001b). Coverslips were crushed using a clean glass stirring rod, and contents subsequently filtered through 47-mm 0.45-μm pore-size Whatman cellulose nitrate filters under gentle vacuum. Filters were then treated and counted as noted above for pelagic PP. Biofilm PP rates were calculated as described above for pelagic samples except that rates were based on the area of the coverslip not the volume filtered.

4.2.5.2 Measurement of bacterial productivity (BP)

The setup for BP incubation was similar to PP except that the incubation was carried out in 50-mL Falcon tubes instead of crystallization dishes. At each sampling time (8, 15, and 21 days), coverslips were harvested randomly and each placed into a Falcon tube containing 20 mL filter sterilized water and 334 μL methyl ^{3}H-thymidine solution (20 nM) (Perkin Elmer). Control tubes received 2 mL formalin. Tubes were incubated *in situ* for 1 h. Thymidine incorporation was stopped by adding 2 mL of 5N NaOH followed by 2 mL formalin. DNA was extracted in the

laboratory as described above except that 2.0 mL of 100 % TCA was added to each sample. BP rates were calculated as for pelagic samples except that the coverslip area was used instead of volume filtered (Waiser, 2001b).

4.2.5.3 Estimation of phytoplankton biomass

Four coverslips harvested from each enclosure were each placed in 50-mL centrifuge tubes containing 10 mL of 90 % ethanol. Tubes were placed on ice and transported back to laboratory. All coverslips were then crushed, samples filtered and Chl *a* extracted and measured as described in pelagic section.

4.2.5.4 Bacterial community structure analysis

Assays of bacterial community structure were conducted using BIOLOG EcoPlates, a technique for testing bacterial carbon source utilization from naturally occurring communities (Garland and Mills, 1991). For these analyses, coverslips from treated and control enclosures were each placed in a 50-mL centrifuge tube containing 5 mL of 0.2-μm filter sterilized water. Samples were stored on ice and transported to the laboratory. Coverslips were crushed using a clean glass rod, contents vortexed and then centrifuged at 3000 g for 5 min. 100 μL of the supernatant was pipetted into each well of a 96 well BIOLOG EcoPlate (BIOLOG Inc., Hayward, CA, USA). One BIOLOG EcoPlate was used for each enclosure for each of the 3 sampling times. Inoculated plates were incubated at 22 ^{o}C and absorbance read after 7 days on an Emax BIOLOG Microstation plate reader (Molecular Devices, Inc., Sunnyvale, CA, USA) at 590 nm. This technique produces a metabolic footprint of the bacterial community which can be used to compare control and treatment communities.

4.2.6 Statistical Analysis

A one-way analysis of variance (ANOVA) was conducted on each water quality parameter to detect any significant differences between control and treatment enclosures and the respective

pond during the study period. A two-way repeated measures analysis of variance (RM ANOVA) was conducted on each response variable (PP and BP rates, and Chl *a* content) from pelagic communities over time to detect any significant differences between control and treatment enclosures within each pond. In addition, student's t-test was performed on each response variable on Day 28 (last sampling day) from the pelagic communities to detect any significant differences between control and treatment enclosures within each pond at the end of the study period.

A student's *t*-test was performed on each response variable (PP and BP rates, and Chl *a* content) from biofilm communities on each sampling day (Days 7, 14, and 21) to detect any significant differences between control and treatment enclosures within each pond on respective days. A one-way ANOVA was performed on BIOLOG data for each of 31 carbon substrates in BIOLOG EcoPlate to detect differences between control and treatment. The level of significance was $p < 0.1$. All statistical tests were conducted using SAS statistical software package, version 9.1 (SAS Institute Inc., Cary, North Carolina, USA).

4.3 Results

4.3.1 Water Quality Parameters

Temperature, pH, specific conductivity, dissolved oxygen (DO), alkalinity, dissolved organic carbon (DOC), NH_3, nitrate (NO_3^-), nitrite (NO_2^-), total phosphorus (TP), and particulate nitrogen (PN) and carbon (PC), measured weekly, were found to be similar in pond and control and treated enclosures across all four ponds (Table 4.2 and Table 4.3). According to specific conductivity results, P109 was freshwater (specific conductivity <600 μS/cm) while the remaining three ponds, P02, P50, and P67, in the increasing order of their salinity, were saline (specific conductivity >600 μS/cm) (Curtis and Adams, 1995). Except DOC content, all nutrients were higher in P109 compared to the other three ponds. P02 had significantly higher ($p < 0.1$) DOC, NH_3, and TP concentrations than P50 and P67 while nitrite + nitrate, PON, and POC were similar to P50 and P67. DOC, NH_3, and TP concentrations were similar in P50 and P67. In P109, sestonic PN:PC (>140), PaPh:PC (> 20), and PN:PaPh (< 10) indicated that the pelagic microbial communities were nitrogen and phosphorus sufficient. In P02, sestonic PN:PC (< 140) indicated nitrogen deficiency while PaPh:PC (> 20), and PN:PaPh (< 10) indicated phosphorus sufficiency. In P50 and P67, sestonic PN:PC (< 140), PaPh:PC (< 20 and > 10), and PN:PaPh (> 10 and < 20) indicated that the pelagic microbial communities were moderately nitrogen and phosphorus deficient.

Table 4.2 Average temperature, pH, specific conductivity, dissolved oxygen and alkalinity in control enclosures (n = 24), treatment enclosures (n = 24) and pond (n = 8) measured during the study period (28 days). Values are reported as average ± standard deviation, n = 24 (control and treatment), n = 8 (pond).

Enclosure / Pond	Temperature (^{o}C)	pH	Specific Conductivity (μS/cm)	Dissolved Oxygen (mg/L)	Alkalinity as $CaCO_3$ (mg/L)
Pond 109					
Control	20 ± 1	7.4 ± 0.2	440 ± 20	3.6 ± 2.0	202 ± 22
Treatment	20 ± 1	7.6 ± 0.4	450 ± 30	4.7 ± 2.5	216 ± 20
Pond	20 ± 1	7.4 ± 0.2	500 ± 20	4.8 ± 2.1	242 ± 13
Pond 02					
Control	21 ± 1	8.0 ± 0.1	3540 ± 130	3.1 ± 1.2	606 ± 14
Treatment	20 ± 1	8.0 ± 0.1	3530 ± 130	3.2 ± 1.3	597 ± 32
Pond	21 ± 1	8.3 ± 0.1	3600 ± 180	4.3 ± 0.7	662 ± 28
Pond 50					
Control	20 ± 1	8.5 ± 0.2	3950 ± 170	7.5 ± 3.2	568 ± 44
Treatment	20 ± 1	8.5 ± 0.2	3900 ± 160	6.5 ± 3.0	521 ± 73
Pond	21 ± 1	8.5 ± 0.1	3880 ± 130	6.3 ± 0.7	529 ± 13
Pond 67					
Control	20 ± 1	8.7 ± 0.2	4790 ± 300	8.5 ± 1.3	459 ± 81
Treatment	20 ± 1	8.9 ± 0.1	4540 ± 190	7.6 ± 0.6	365 ± 20
Pond	21 ± 1	8.8 ± 0.1	4540 ± 200	7.4 ± 0.8	344 ± 17

Table 4.3 Average dissolved organic carbon (DOC), NH_3, nitrite and nitrate, total phosphorus (TP), and particulate organic nitrogen and carbon in control enclosures (n = 24), treatment enclosures (n = 24) and pond (n = 8) measured during the study period (28 days). Values are reported as average ± standard deviation, n = 24 (control and treatment), n=8 (pond).

Enclosure / Pond	DOC (mg/L)	NH_3 (mg/L)	$NO_2 + NO_3$ (mg/L)	TP (mg/L)	PON (mg/L)	POC (mg/L)
Pond 109						
Control	24 ± 1	0.86 ± 1.01	<0.01	1.42 ± 0.29	0.31 ± 0.39	1.48 ± 1.11
Treatment	26 ± 1	0.47 ± 0.41	<0.01	1.70 ± 0.29	0.43 ± 0.34	1.88 ± 1.34
Pond	26 ± 2	0.20 ± 0.21	<0.01	2.18 ± 0.32	0.88 ± 0.32	5.24 ± 2.38
Pond 02						
Control	83 ± 4	0.6 ± 0.35	0.02 ± 0.01	0.74 ± 0.14	0.08 ± 0.04	0.62 ± 0.28
Treatment	83 ± 4	0.5 ± 0.34	0.02 ± 0.01	0.69 ± 0.09	0.13 ± 0.10	0.74 ± 0.30
Pond	85 ± 6	0.2 ± 0.04	0.02 ± 0.01	0.88 ± 0.12	0.09 ± 0.04	0.59 ± 0.20
Pond 50						
Control	52 ± 3	0.05 ± 0.02	<0.01	0.09 ± 0.01	0.14 ± 0.10	0.81 ± 0.24
Treatment	51 ± 2	0.08 ± 0.06	<0.01	0.12 ± 0.03	0.12 ± 0.05	0.92 ± 0.39
Pond	47 ± 2	0.06 ± 0.04	<0.01	0.10 ± 0.00	0.24 ± 0.20	1.02 ± 0.70
Pond 67						
Control	50 ± 5	0.06 ± 0.02	<0.01	0.06 ± 0.01	0.11 ± 0.10	0.46 ± 0.12
Treatment	47 ± 4	0.07 ± 0.03	<0.01	0.07 ± 0.00	0.11 ± 0.07	0.65 ± 0.11
Pond	45 ± 4	0.07 ± 0.04	<0.01	0.08 ± 0.00	0.20 ± 0.08	1.68 ± 0.70

4.3.2 Pelagic Communities

4.3.2.1 Pond 109 (P109)

Pelagic PP over time was similar between control and treated enclosures (RM ANOVA, $p > 0.1$) (Figure 4.4A). PP in control and treated enclosures were similar on Day -3; however, greater increases in rates were observed in treated enclosure on Days 1 and 2. PP was similar in control and treatment at the end of the study (*t*-test, $p > 0.1$). Chl *a* did not show any significant difference between control and treatment enclosures over time during the study period (RM ANOVA, $p = 0.8583$) (Figure 4.5A). Chl *a* content was similar in control and treatment on Day 28, end of the study (*t*-test, $p > 0.1$). Sestonic protein to carbohydrate ratios indicated that phytoplankton were not nutrient-deficient in both control and treated enclosures (Figure 4.6A).

Pelagic bacterial productivity (BP) over time was significantly different for treated and control enclosures (RM ANOVA, $p = 0.0004$) (Figure 4.7A). BP increased sharply in treatment on Day 7 compared to control, while those were similar both control and treatment from Day 14 till the end of the study.

4.3.2.2 Pond 02 (P02)

Pelagic PP over time was similar when treated and control enclosures were compared (RM ANOVA, $p = 0.2901$) (Figure 4.4B). PP increased in treated enclosures from Day 7 till the end of the study period. Rates were significantly higher in treatment than control on Day 28 (*t*-test, $p < 0.1$). Chl *a* content over time was similar in control and treated enclosures (RM ANOVA, $p = 0.4622$) (Figure 4.5B). Phytoplankton showed moderate to severe nutrient deficiency over the study period in both control and treatment enclosures as indicated by protein to carbohydrate ratios (Figure 4.6B).

Pelagic BP over time was significantly different for treated and control enclosures (RM ANOVA, $p = 0.0008$) (Figure 4.7B). During the study period, BP was highest in control on Day 7, while in treated enclosures, on Day 14. BP was similar in both control and treated enclosures on day 28 (t-test, $p > 0.1$).

4.3.2.3 Pond 50 (P50)

Pelagic PP over time was similar when treated and control enclosures were compared (RM ANOVA, $p = 0.1473$) (Figure 4.4C). Rates in both control and treatment were similar until Day 7. Thereafter PP was higher in treatments from Day 14 until the end of the study. Rates were significantly higher in treatment compared to control on Day 28 (*t*-test, $p < 0.1$). Chl *a* over time was significantly different in the treatment when compared to the control (RM ANOVA, $p = 0.0470$) (Figure 4.5C). Chl *a* was greater in the treatment compared to control on Day 21, but by day 28, Chl *a* in both treatment and control was similar. As indicated by protein to carbohydrate ratios, phytoplankton showed moderate to severe nutrient deficiency in control enclosures, while phytoplankton in treated enclosures were moderately nutrient deficient during study period (Figure 4.6C).

Pelagic BP over time was significantly different for treated and control enclosures (RM ANOVA, $p = 0.0218$) (Figure 4.7C). Although BP in treatment and control followed a similar pattern on Days 7, 14, and 21, rates were significantly lower in treatment compared to control on Day 28 (*t*-test, $p < 0.1$).

4.3.2.4 Pond 67 (P67)

Pelagic PP over time was significantly different in treated enclosure when compared to controls (RM ANOVA, $p = 0.0164$) (Figure 4.4D). PP followed a similar pattern in both control and treatment enclosures until Day 7. Thereafter an increase in rates was observed on Days 14 and 28 in treatment when compared to control. Rates were significantly higher in treatment compared to control on Day 28 (*t*-test, $p < 0.1$). Chl *a* over time was similar in treatment when compared to control (RM ANOVA, $p = 0.1908$) (Figure 4.5D). Chl *a* followed a similar pattern in both control and treated enclosures for the duration of the study. Phytoplankton showed moderate to severe nutrient deficiency during the study period in both control and treated enclosures as suggested by protein to carbohydrate ratios (Figure 4.6D).

Over time pelagic BP was significantly different in treatment when compared to control (RM ANOVA, $p = 0.0375$) (Figure 4.7D). Although there was increase in BP in treatment on Day 1 and 2, rates were similar to control from Day 3 until the end of the study.

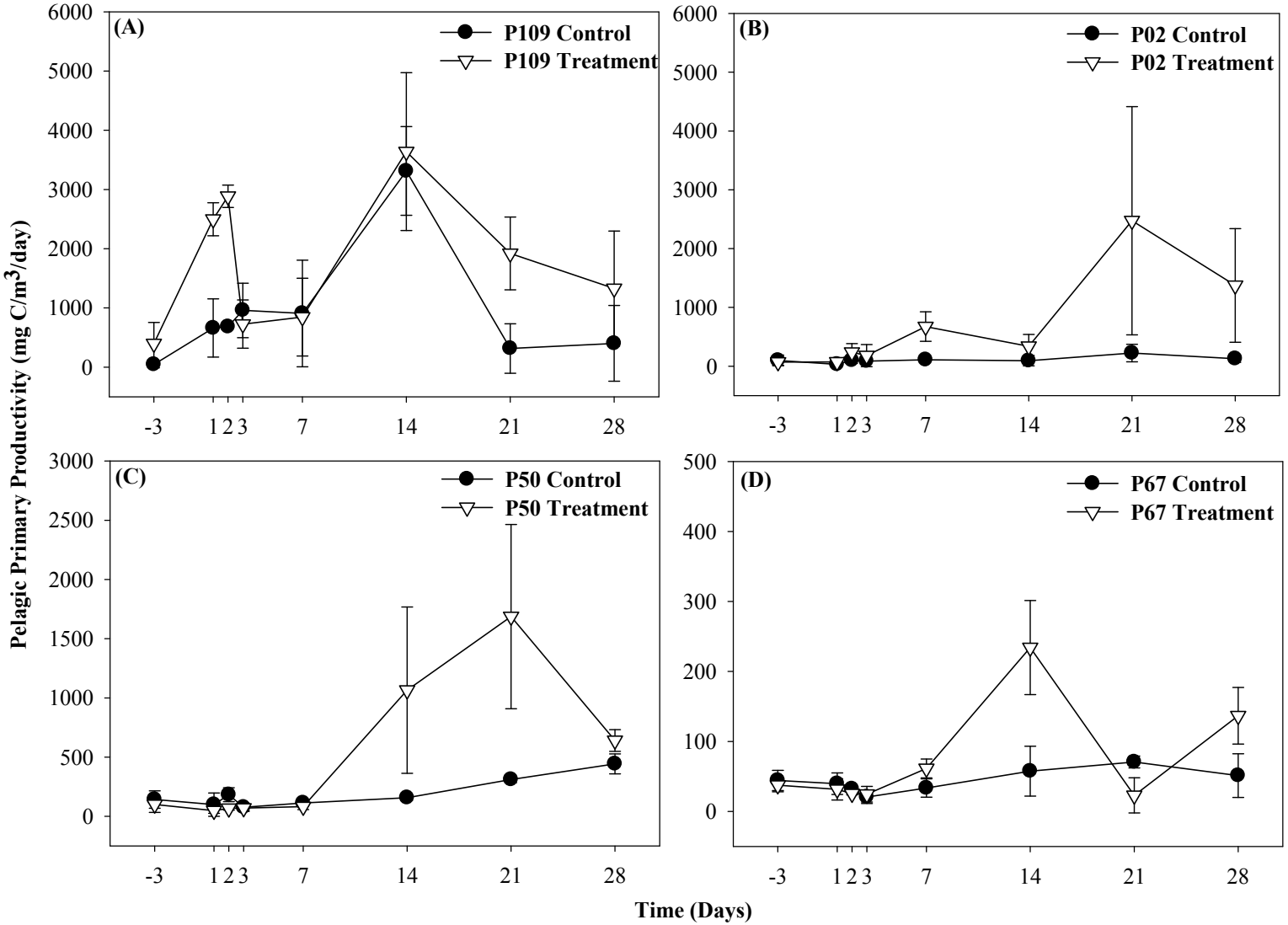


Figure 4.4 Pelagic primary productivity (PP) in control and treated enclosures in pond 109 (A), pond 02 (B), pond 50 (C) and pond 67 (D). Error bars represent SD, n=3.

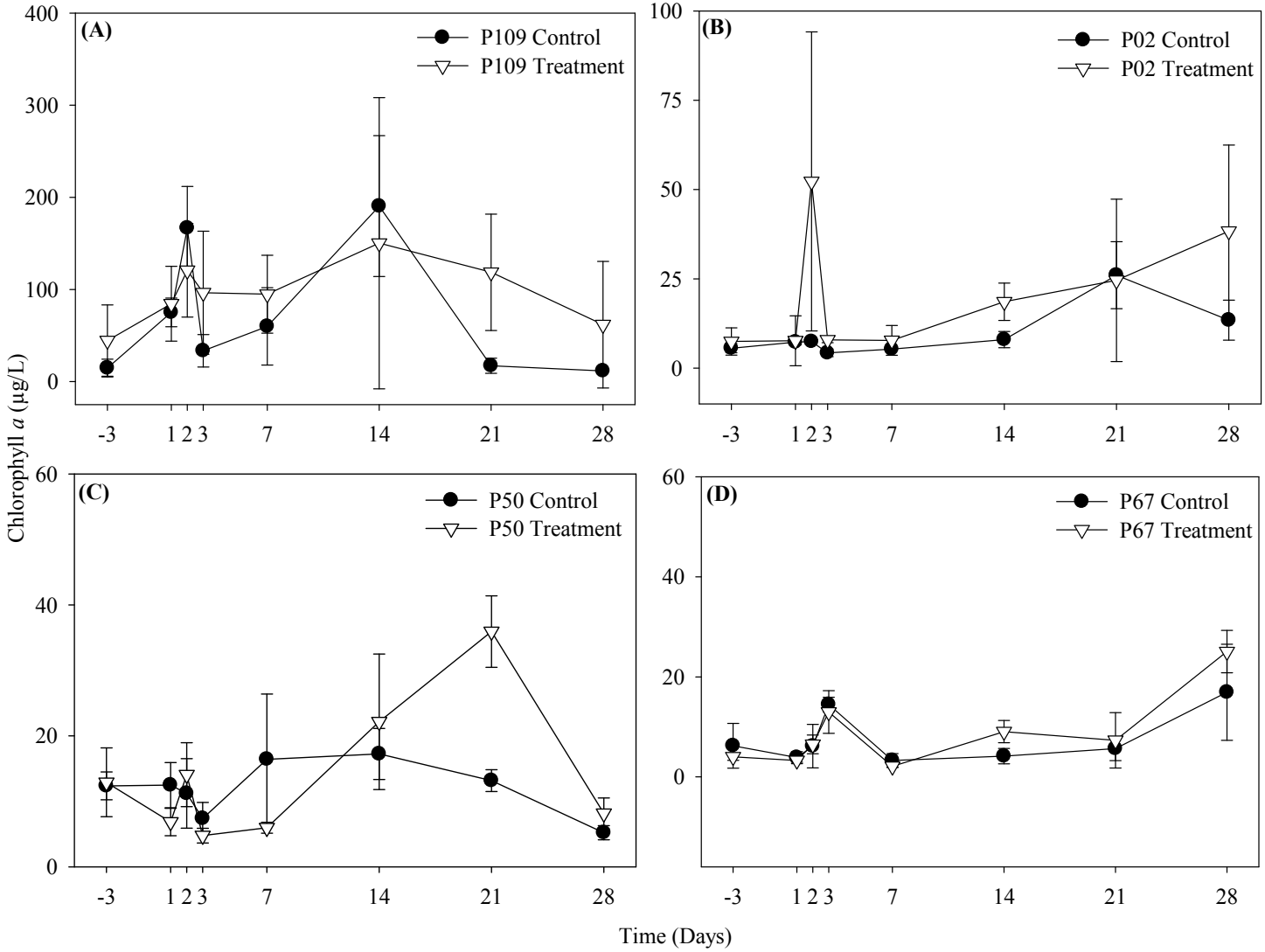


Figure 4.5 Pelagic chlorophyll *a* (Chl *a*) concentrations in control and treated enclosures in pond 109 (A), pond 02 (B), pond 50 (C) and pond 67 (D). Error bars represent SD, n=3.

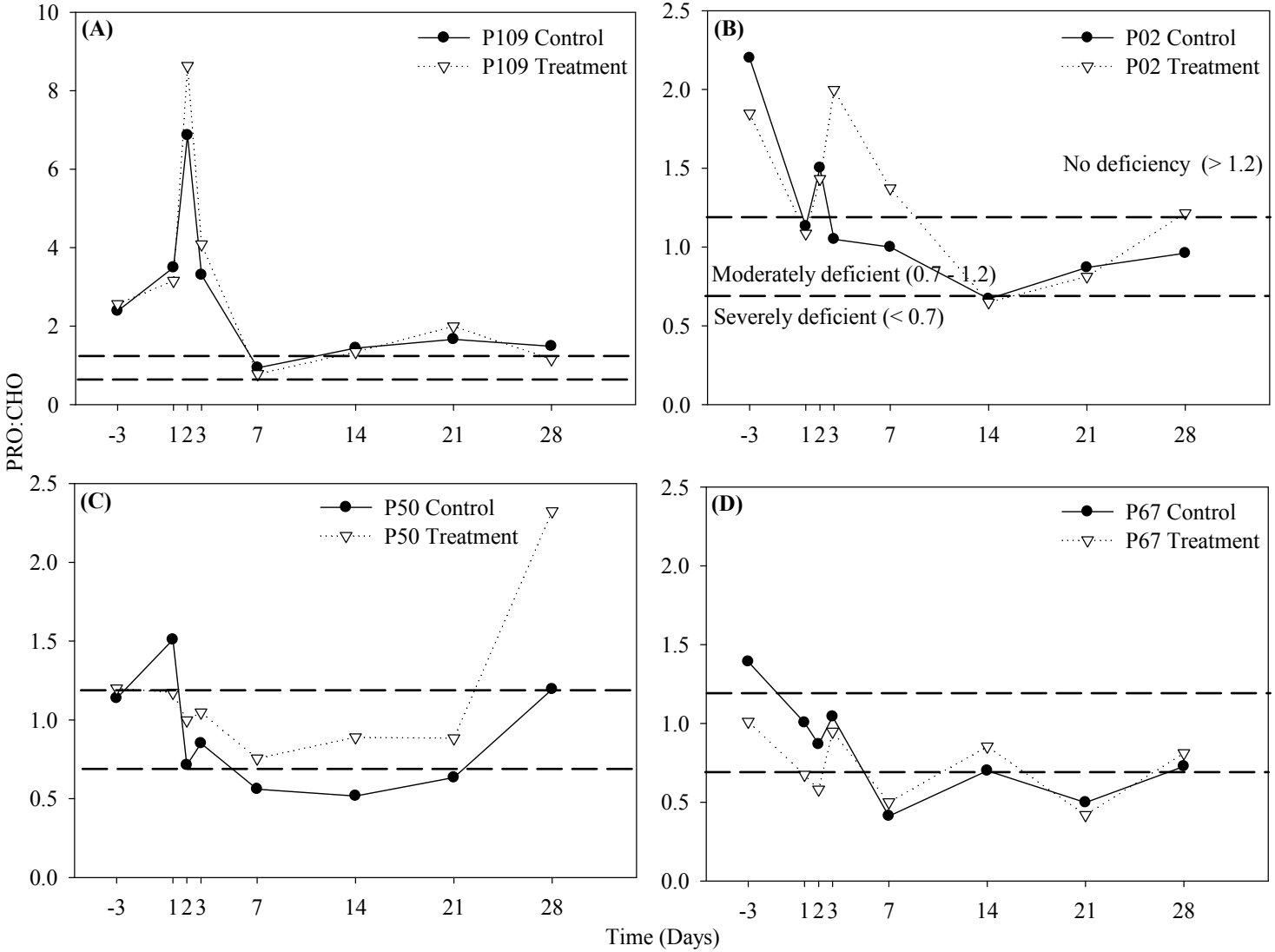


Figure 4.6 Protein (PRO) to carbohydrate (CHO) ratios in control and treated enclosures in pond 109 (A), pond 02 (B), pond 50 (C), and pond 67 (D). Threshold lines (broken lines) indicate nutrient status of algae (ratios >1.2 (no deficiency); 0.7 to 1.2 (moderately deficient); <0.7 (severely deficient)).

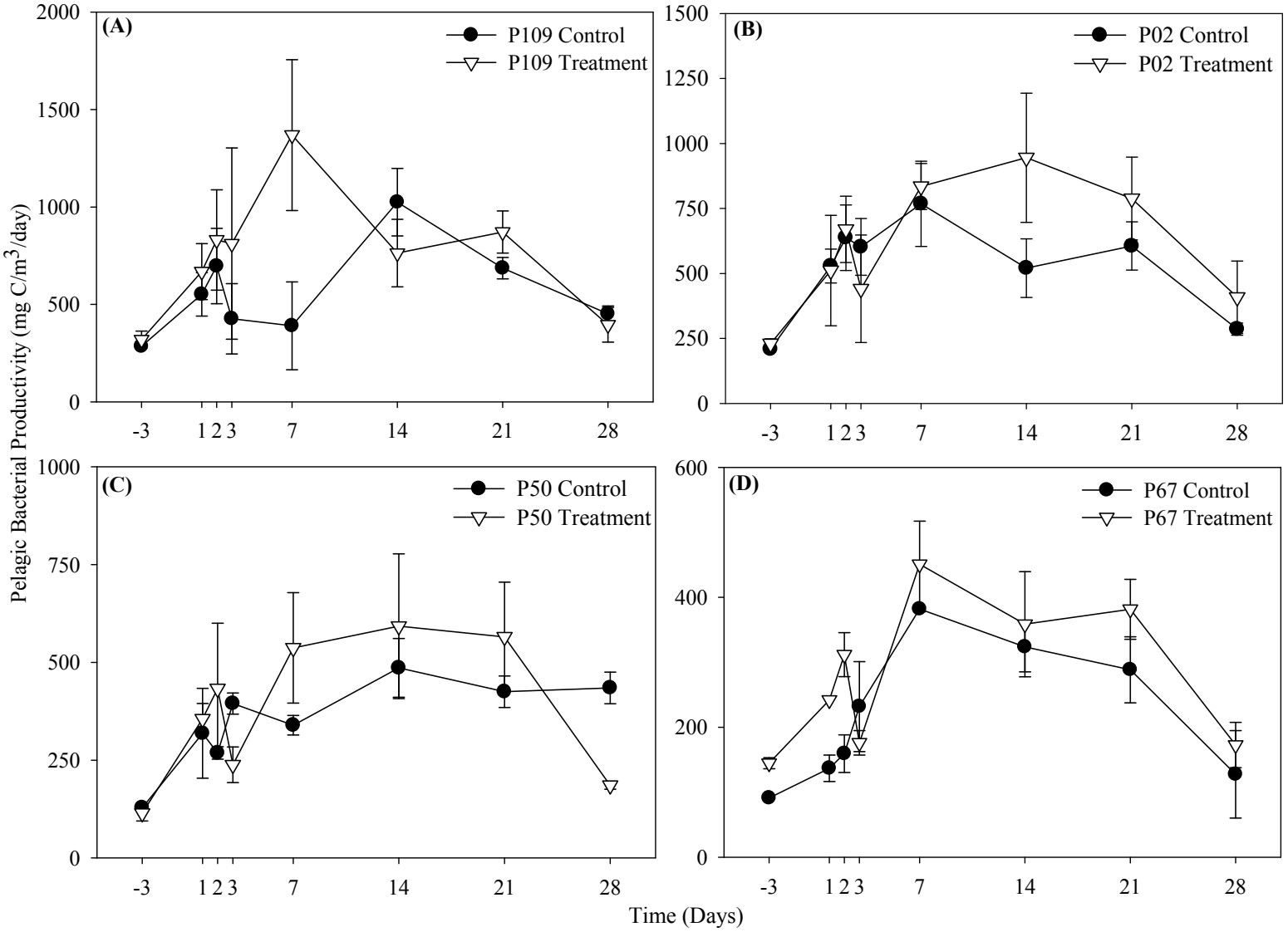


Figure 4.7 Pelagic bacterial productivity (BP) in control and treated enclosures in pond 109 (A), pond 02 (B), pond 50 (C) and pond 67 (D). Error bars represent SD, n=3.

4.3.3 Biofilm Communities

4.3.3.1 Pond 109 (P109)

Biofilm PP was similar in treated and control enclosures on all three sampling days (*t*-test, $p > 0.1$) (Figure 4.8A). Chl *a* was similar in treated and control enclosures on Days 7 and 21 (*t*-test, $p > 0.1$) while significantly higher in treated than control enclosures on Day 14 (*t*-test, $p < 0.1$) (Figure 4.9A). BP showed no significant differences between treated and control enclosures on all sampling days (*t*-test, $p > 0.1$) (Figure 4.10A). BIOLOG data indicated that on Day 7 carbon utilization was significantly different in 4 of 31 carbon substrates, on Day 14 in 9 substrates and on Day 21, in 4 substrates when control and treatments were compared (Table 4.4).

4.3.3.2 Pond 02 (P02)

Biofilm PP, Chl *a*, and BP were similar in treated and control enclosures on all three sampling days (*t*-test, $p > 0.1$) (Figure 4.8B, Figure 4.9B, Figure 4.10B). BIOLOG data indicated that on Day 7 carbon utilization was significantly different in 7 of 31 carbon substrates, on Day 14 in 4 substrates and on Day 21, in 6 substrates when control and treatment biofilms were compared (Table 4.4).

4.3.3.3 Pond 50 (P50)

Biofilm PP, Chl *a*, and BP were similar in treated and control enclosures on all three sampling days (*t*-test, $p > 0.1$) (Figure 4.8C, Figure 4.9C, Figure 4.10C). BIOLOG data indicated that on Day 7 carbon utilization was significantly different in 3 of 31 carbon substrates and on Day 21, four substrates, while no difference on Day 14, when control and treatment biofilms were compared (Table 4.5).

4.3.3.4 Pond 67 (P67)

Biofilm PP was similar in treated and control enclosures on Days 7 and 14 (*t*-test, $p > 0.1$), while rates were higher in treatment than control on Day 21 (Figure 4.8D). Chl *a* was similar in

treated and control enclosures on Days 7, and 14 (*t*-test, $p > 0.1$) but significantly higher in treated enclosure compared to control on Day 21 (*t*-test, $p < 0.1$) (Figure 4.9D). No significant differences in BP were noted between treated and control enclosures on all sampling days (*t*-test, $p > 0.1$) (Figure 4.10D). BIOLOG data indicated that on Day 7 carbon utilization was significantly different in 3 of 31 carbon substrates, on Day 14 in 1 substrate, while no difference was observed on Day 21 when control and treatment biofilms were compared (Table 4.5).

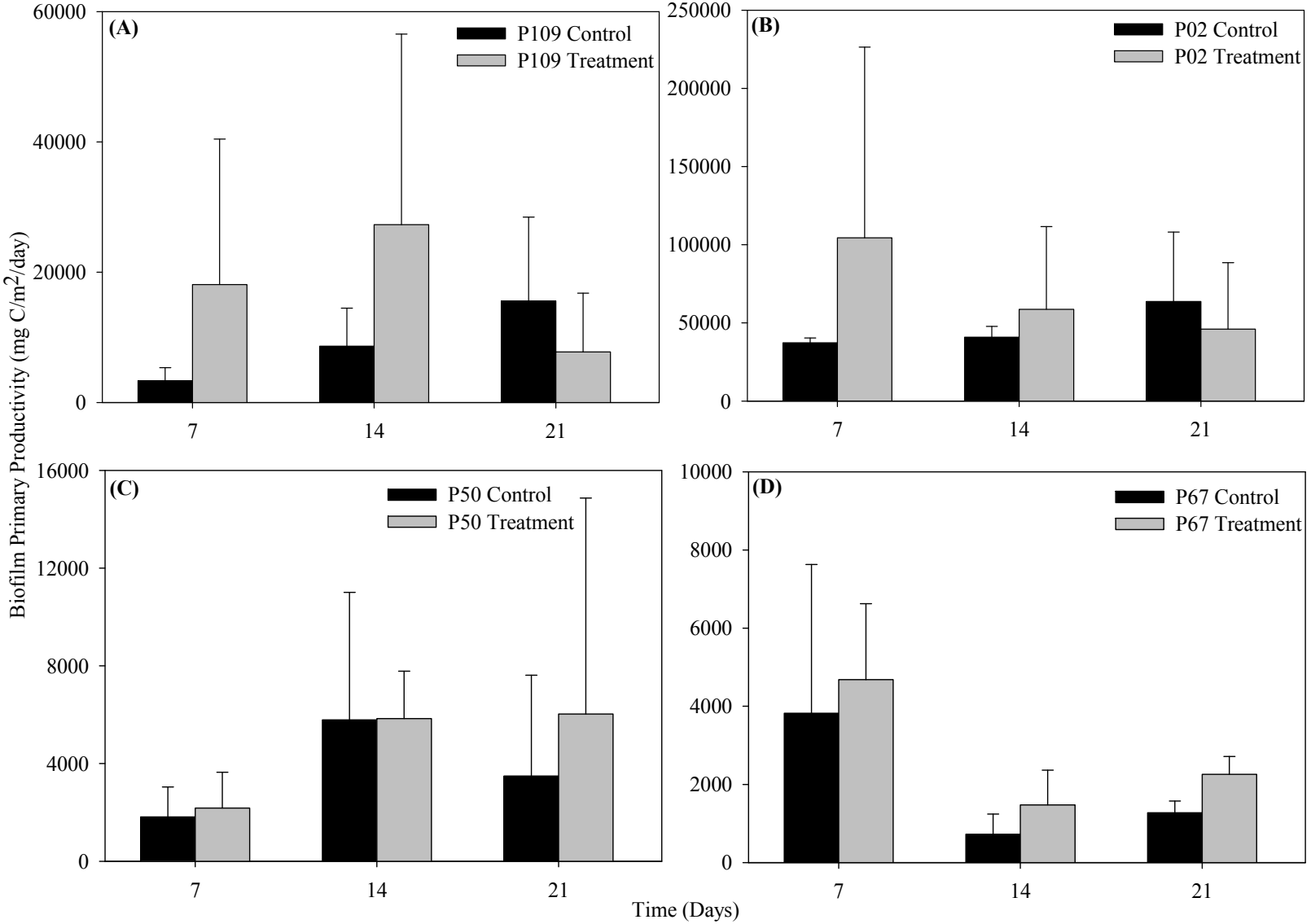


Figure 4.8 Biofilm primary productivity (PP) in control and treated enclosures in pond 109 (A), pond 02 (B), pond 50 (C) and pond 67 (D). Error bars represent SD, n=9.

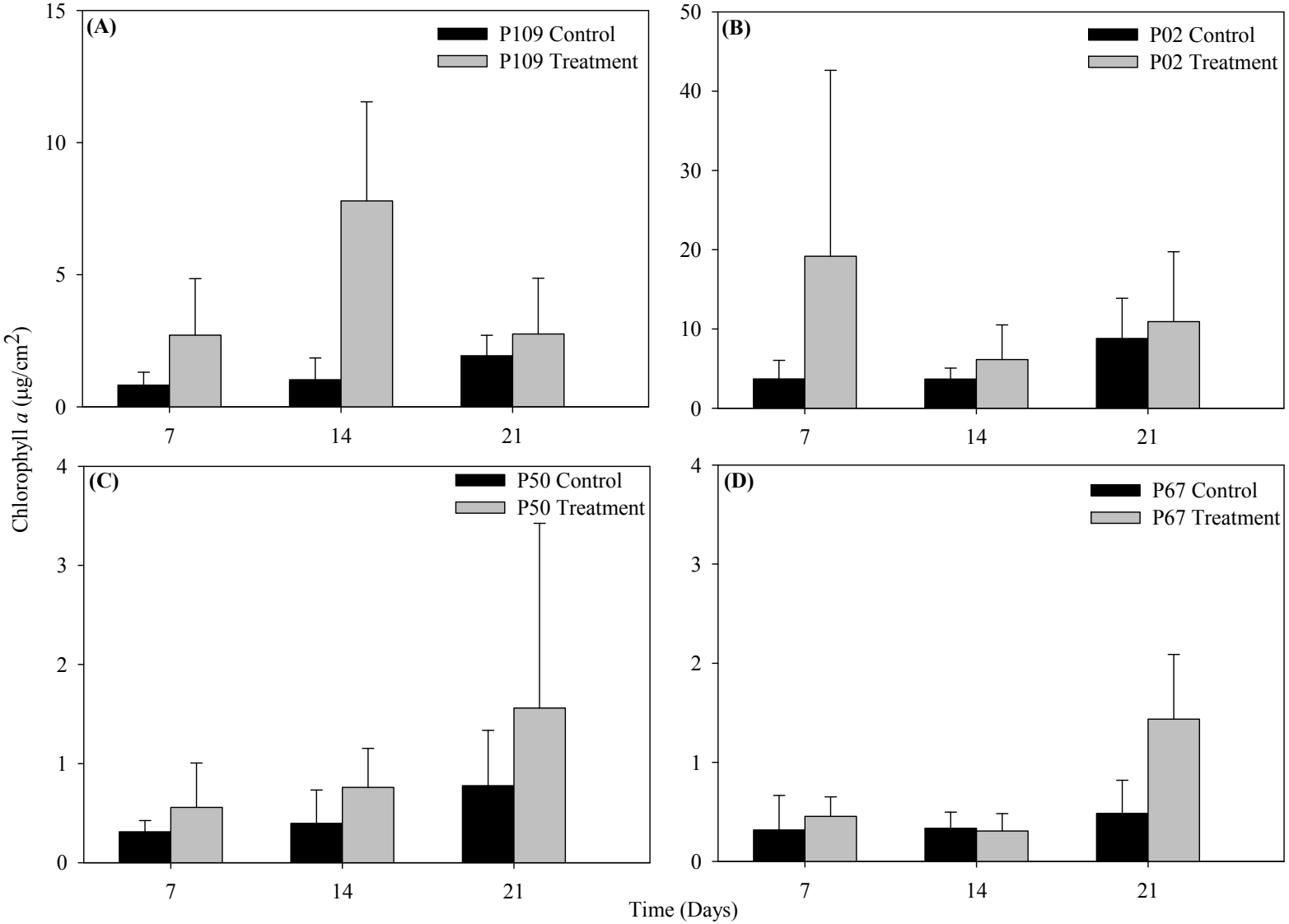


Figure 4.9 Biofilm chlorophyll *a* (Chl *a*) concentrations in control and treated enclosures in pond 109 (A), pond 02 (B), pond 50 (C) and pond 67 (D). Error bars represent SD, n=9.

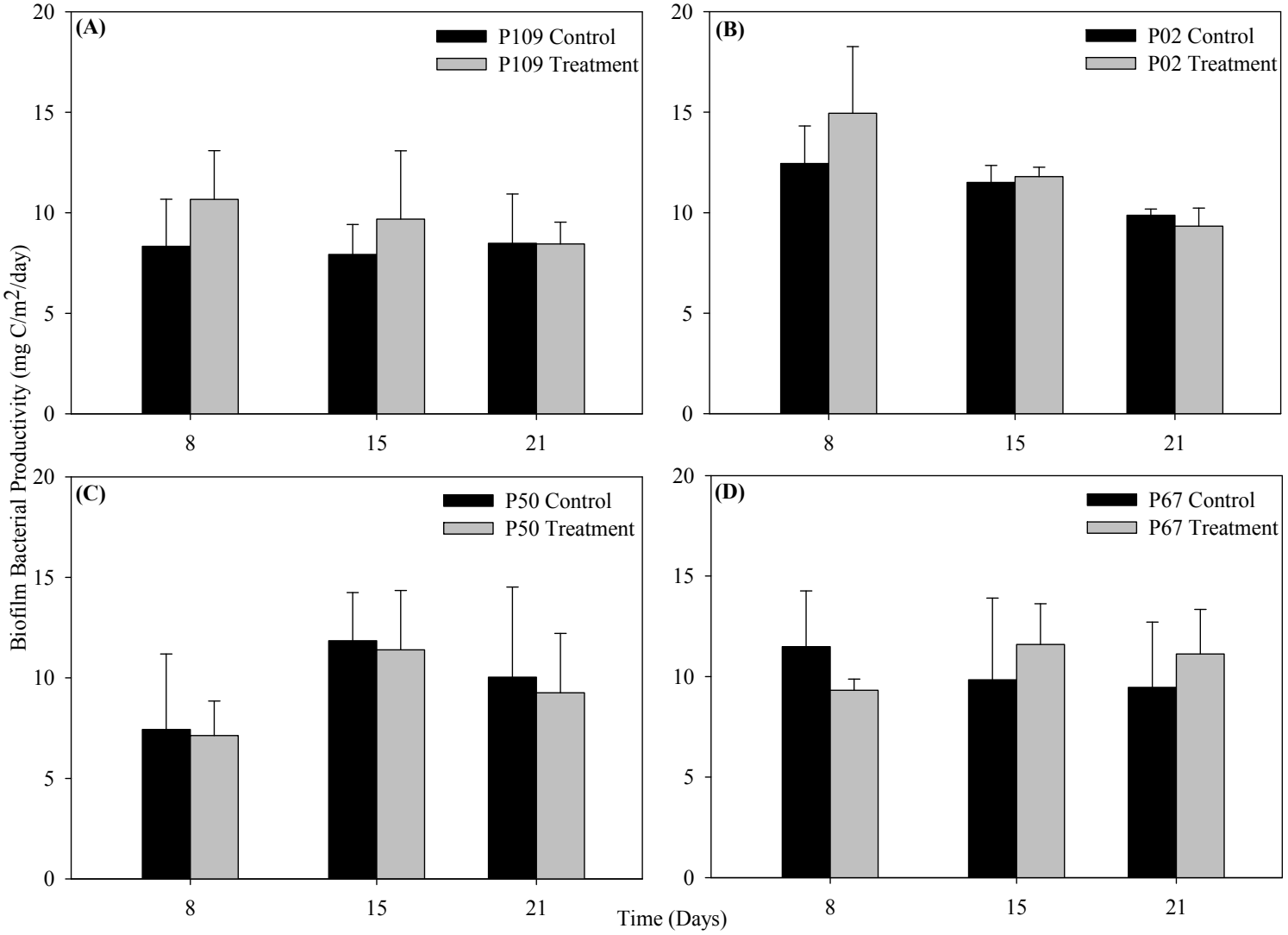


Figure 4.10 Biofilm bacterial productivity (BP) in control and treated enclosures in pond 109 (A), pond 02 (B), pond 50 (C) and pond 67 (D). Error bars represent SD, n=9.

Table 4.4 Summary statistics of Biolog plate incubations of biofilm samples collected from enclosures on Days 8, 16, and 23 from Ponds 109 and 02. D denotes significant difference between control (C) and Treatment (T) within wetland on the same day. ($p < 0.05$).

Carbon sources in the Biolog Eco-plate	Pond 109						Pond 02					
	Day 8		Day 15		Day 22		Day 8		Day 15		Day 22	
	C	T	C	T	C	T	C	T	C	T	C	T
POLYMER												
α-Cyclodextrin	D	D			D	D						
Tween 40									D	D	D	D
Tween 80							D	D				
CARBOHYDRATE												
D-Xylose							D	D			D	D
i-Erythritol												
Glycogen												
ß-Methyl-D-Glucoside			D	D								
N-Acetyl-D-Glucosamine			D	D								
D-Cellobiose									D	D	D	D
α-D-Lactose												
D-Mannitol							D	D			D	D
CARBOXYLIC ACID												
2-Hydroxy Benzoic Acid	D	D							D	D		
α-Ketobutyric Acid	D	D										
Itaconic acid												
D-Malic Acid			D	D								
D-Galactonic Acid γ-Lactone			D	D			D	D				
D-Glucosaminic Acid												
4-Hydroxy Benzoic Acid											D	D
γ-Hydroxybutyric Acid												
D-Galacturonic Acid	D	D					D	D				
AMINO ACID												
L-Threonine							D	D				
Glycyl-L-Glutamic Acid			D	D	D	D						
L-Phenylalanine									D	D	D	D
L-Serine			D	D								
L-Arginine												
L-Asparagine					D	D						
AMINE												
Putrescine												
Phenylethylamine			D	D			D	D				
PHOSPHORYLATED												
D,L-α-Glycerol Phosphate			D	D	D	D						
Glucose-1-Phosphate			D	D								
ESTER												
Pyruvic Acid Methyl Ester												

Table 4.5 Summary statistics of Biolog plate incubations of biofilm samples collected from enclosures on Days 8, 16, and 23 from Ponds 50 and 67. D denotes significant difference between control (C) and Treatment (T) within wetland on the same day. ($p < 0.05$).

Carbon sources in the Biolog Eco-plate	Pond 50						Pond 67					
	Day 8		Day 15		Day 22		Day 8		Day 15		Day 22	
	C	T	C	T	C	T	C	T	C	T	C	T
POLYMER												
α-Cyclodextrin							D	D				
Tween 40	D	D							D	D		
Tween 80	D	D										
CARBOHYDRATE												
D-Xylose												
i-Erythritol												
Glycogen												
ß-Methyl-D-Glucoside												
N-Acetyl-D-Glucosamine												
D-Cellobiose												
α-D-Lactose							D	D				
D-Mannitol												
CARBOXYLIC ACID												
2-Hydroxy Benzoic Acid					D	D						
α-Ketobutyric Acid												
Itaconic acid					D	D						
D-Malic Acid												
D-Galactonic Acid γ-Lactone					D	D						
D-Glucosaminic Acid												
4-Hydroxy Benzoic Acid												
γ-Hydroxybutyric Acid	D	D			D	D						
D-Galacturonic Acid												
AMINO ACID												
L-Threonine												
Glycyl-L-Glutamic Acid												
L-Phenylalanine												
L-Serine												
L-Arginine							D	D				
L-Asparagine												
AMINE												
Putrescine												
Phenylethylamine												
PHOSPHORYLATED												
D,L-α-Glycerol Phosphate												
Glucose-1-Phosphate												
ESTER												
Pyruvic Acid Methyl Ester												

4.4 Discussion

4.4.1 Pelagic Communities

The mixture of eight herbicides used in the present study represented a maximum-exposure scenario. These herbicide concentrations were 1.1 times (for glyphosate) to 49 times (for MCPA) greater than the respective Canadian Water Quality Guidelines for the Protection of Aquatic Life (Table 4.1). At this total concentration, the herbicide mixture had differing effects on microbial communities in ponds which varied from fresh to saline. While immediate effects on PP were noted in the freshwater pond similar effects were not observed until 7 to14 days after treatment in the three saline ponds.

Although PP was stimulated in freshwater pond, P109, immediately following treatment (Day 1 and 2) no such stimulation of algal biomass was noted. Stimulation of PP was similar to observations made in a curtained wetland study after an ephemeral freshwater wetland was treated with the same herbicide mixture and at similar concentrations to the present study (Sura et al., 2012, accepted for publication). Such stimulation may be due to the mode of action (MOA) of six of the eight herbicides in the mixture (2,4-D, MCPA, clopyralid, dicamba, mecoprop and dichlorprop). These herbicides have similar MOA to those of naturally occurring auxins (eg., indole-3-acetic acid). Auxin-type herbicides induce synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase via increased expression of specific ACC synthase genes (Abel and Theologis, 1996; Kende and Zeevaart, 1997; Wei et al., 2000). ACC synthase is an important enzyme in the ethylene biosynthesis pathway and catalyses the conversion of *S*-adenosylmethionine to ACC which, in turn, forms ethylene. Ethylene, a plant hormone, is responsible for plant growth regulation, epinasty, senescence, and stimulation of abscisic acid (ABA) production (Kende and Zeevaart, 1997). Although auxins, ethylene, and ABA acting together may bring about the death of plant tissue at higher concentrations, at low

concentrations, growth by cell division and cell elongation is usually stimulated (Grossmann, 2000). Such stimulatory effects by toxic substances at low and sub lethal concentrations are similar to the commonly observed phenomenon, hormesis (Stebbing, 1982). With regard to effects of auxin-type herbicides, they have been found to be stimulatory at the concentrations used in this study. For example, 2,4-D stimulated algal growth and production at 200 μg/L (Wong, 2000) and at 2000 μg/L (Boyle, 1980; Kobraei and White, 1996). According to the concept of concentration addition, the total effect of the six auxin-type herbicides can be reasonably predicted based on their additive toxicity (Faust et al., 2001). In the present study, the auxin-type herbicide concentration was 662 μg/L (target concentration), which is in the range where stimulation of algal growth has been reported in the literature as mentioned above.

The other two herbicides in the mixture are bromoxynil and glyphosate. Bromoxynil, a photosynthesis inhibitor binds to quinine, one of the electron acceptors in photosystem II thereby preventing electron flow to plastoquinone in the electron transport system (Tomaso, 1994), while glyphosate interferes with the shikimic acid pathway disrupting synthesis of aromatic amino acids. The primary target of glyphosate, 5-enolpyruvyl shikimate 3-phosphate synthetase (EPSPase), catalyzes the formation of 5-enolpyruvyl shikimate 2-phosphate (EPSP), an intermediate in the shikimic acid pathway (Boocock and Coggins, 1983). With regard to toxic effects, laboratory studies have revealed that bromoxynil added to distilled water at 280 μg/L (about 4.5 times greater than the target concentration used in the present study) did not inhibit algal growth (Peterson et al., 1994). In another laboratory study using algae cultured in a growth medium, glyphosate at 200 μg/L (about 2.8 times greater than the target concentration used in the present study) had no effect, whereas at 20 μg/L (about one-third the target concentration used in the present study) stimulation of algal growth, photosynthetic rate and Chl *a* content was

noted (Wong, 2000). Glyphosate inhibited algal growth, photosynthetic rate and Chl *a* at concentrations of 2000 µg/L and greater. In another laboratory study, Peterson et al. (1994) investigated glyphosate effects on 11 algal species and determined that 2848 µg/L glyphosate inhibited ^{14}C uptake by 73-77 % in two algal species, while in another two species, only 3-18 % inhibition was noted. Based on this evidence, the two herbicides, bromoxynil and glyphosate were likely present at concentrations too low to cause a negative effect on pelagic phytoplankton in treated enclosures of freshwater pond. Glyphosate, however, may have been in the range where it caused stimulation in conjunction with the auxin-type herbicides.

Based on observations in the freshwater pond (P109), similar stimulation of PP was expected in the other three ponds. Unlike the freshwater pond, however, such stimulation was not observed in the saline ponds during the first week after treatment. It may be that water chemistry and quality parameters, (pH, salinity and nutrients) influenced the ability of the herbicide mixture to affect microbial communities. Salinity, pH, nutrient concentrations, and chemical form of the pesticides in the aquatic ecosystems may alter the response of the microbial community (DeLorenzo et al., 2001). Waiser and Robarts (1997), working on a magnesium sulphate saline lake (Redberry Lake, Saskatchewan, Canada), for example, observed that when microcosms were treated with triallate herbicide, phytoplankton biomass, and bacterial numbers and productivity increased, but only when microcosms were supplemented with nitrogen and phosphorus. No such effects were observed in control microcosms (no nutrients added). Protein-to-carbohydrate and sestonic ratios in the current study indicate that the three saline ponds were moderately to severely nutrient deficient suggesting that nutrient limitation might have played a role in lack of stimulation noted for the saline ponds in the enclosure study. The stimulatory effect of auxin-type herbicides in these saline ponds was counter acted by lack of nutrients to

support increasing primary productivity. There is a correlation between PP and water quality parameters (specific conductivity and concentrations of major ions including SO_4^{-2} and Mg^{+2} - Figures 4.11, 4.12, and 4.13, respectively) among these four ponds. PP appears to decrease with an increase in specific conductivity, Mg^{+2} and SO_4^{-2}. It appears that the perceived effects of the herbicide mixture are dependent on the water quality parameters such as specific conductivity and concentration of major ions. DeLorenzo et al. (2009) observed that pyrethroid insecticide toxicity in adult grass shrimp decreased with increased salinity and suggested that the insecticide was less bioavailable in higher saline water due to binding, resulting in decreased toxicity. In spite of lack of direct evidence of herbicide toxicity on phytoplankton in saline waters, the basic mechanism of binding may be applicable in the enclosure study saline ponds. Given the high concentrations of major ions in saline ponds of enclosure study, herbicides may be less bioavailable to phytoplankton due to binding to these ions. Further research is required to understand the exact role of these water quality parameters in algal community responses to herbicidal activity.

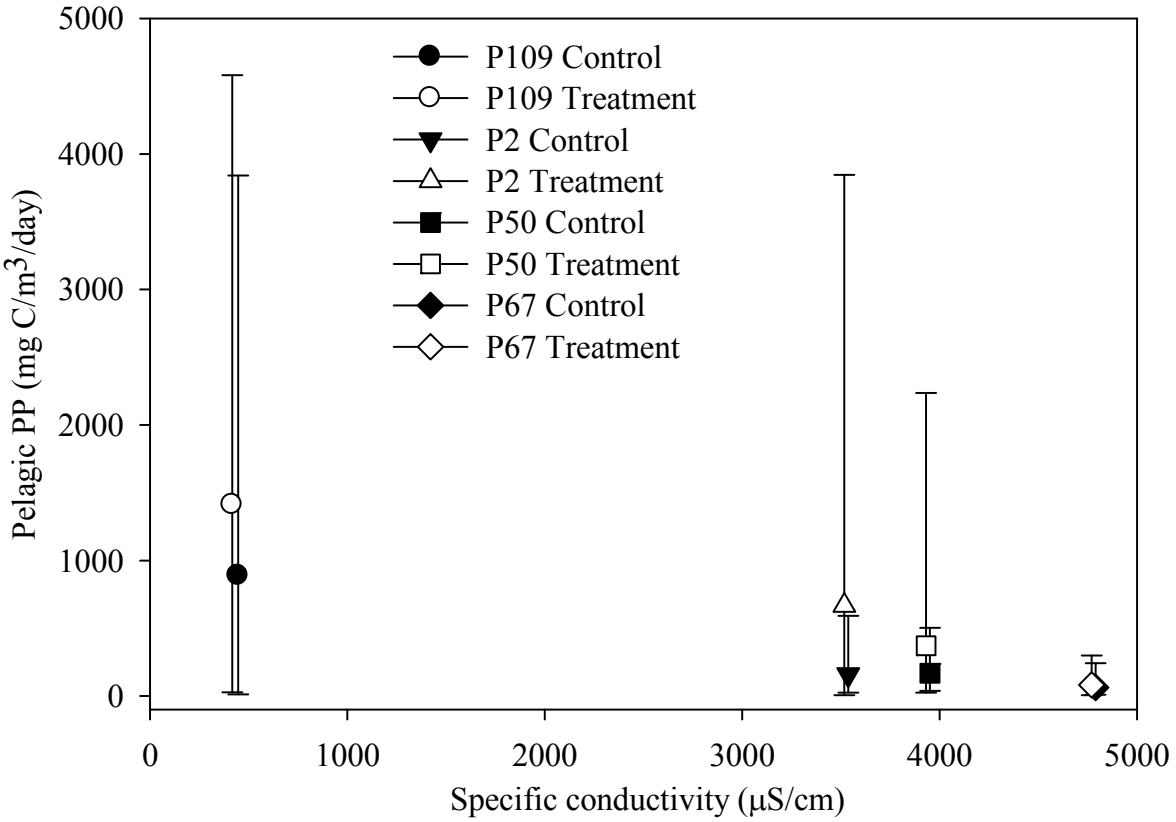


Figure 4.11 Variation of pelagic primary productivity (PP) with specific conductivity. Error bars represent range of PP during the 4-week study period with upper limit indicating maximum PP observed and lower limit indicating minimum PP observed.

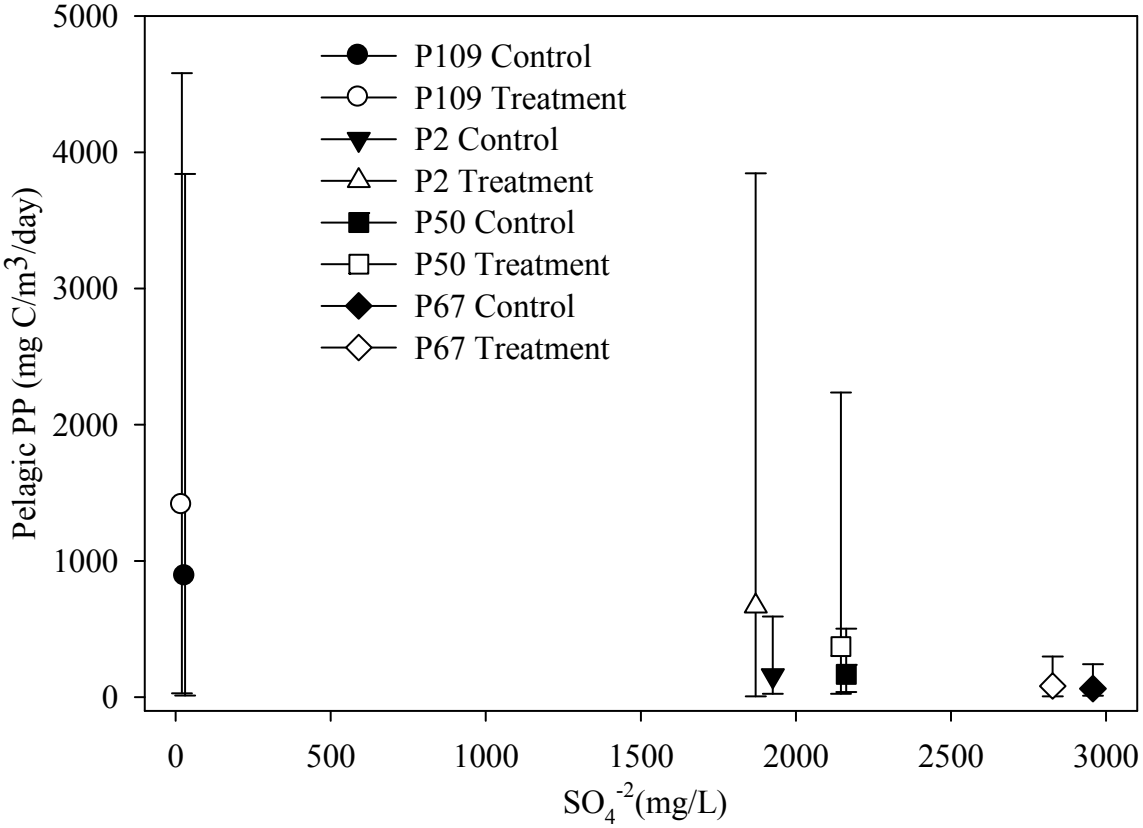


Figure 4.12 Variation of pelagic primary productivity (PP) with sulfate ion concentration. Error bars represent range of PP during the 4-week study period with upper limit indicating maximum PP observed and lower limit indicating minimum PP observed.

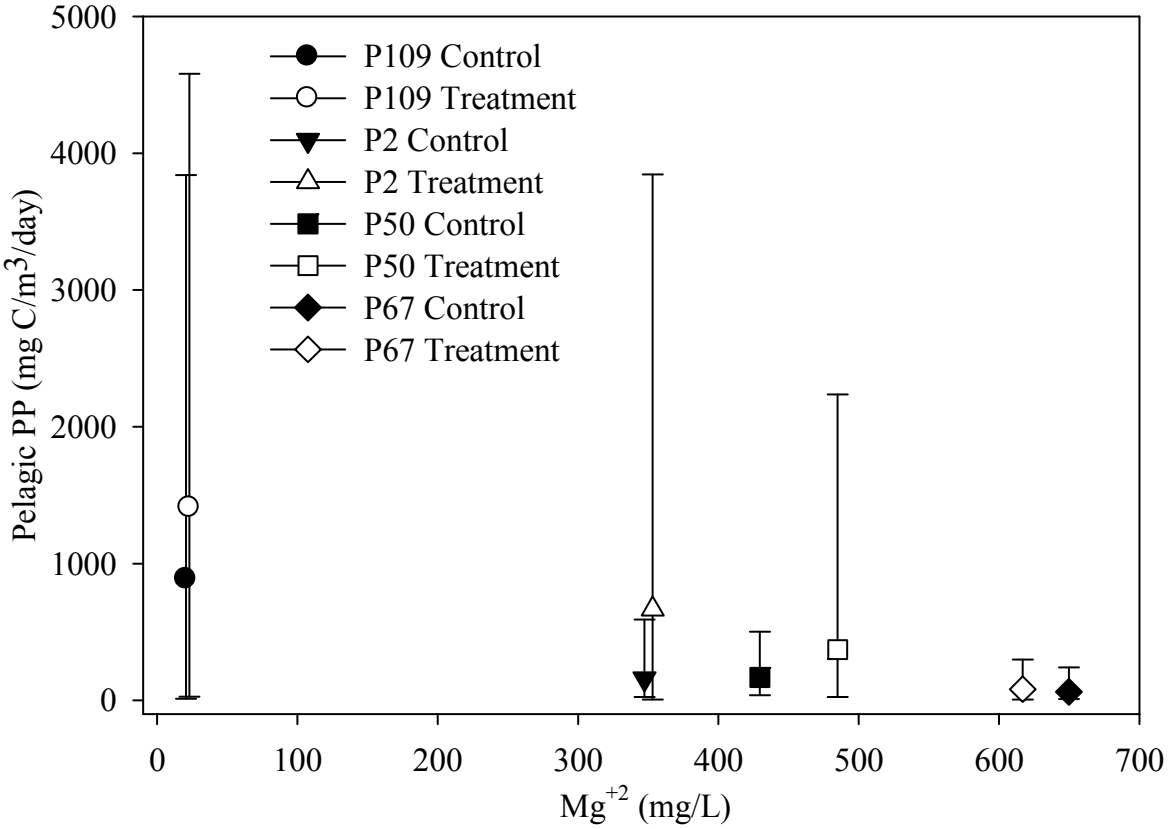


Figure 4.13 Variation of pelagic primary productivity (PP) with magnesium ion concentration. Error bars represent range of PP during the 4-week study period with upper limit indicating maximum PP observed and lower limit indicating minimum PP observed.

4.4.2 Biofilm Communities

Biofilm communities were not affected by the herbicide treatment across all ponds. Although there was a trend indicating increased biofilm PP in P109, such growth was not statistically significant probably because of increased grazing by snails in treated enclosures. Even though the snail population was not enumerated in this study, it was observed that they were significantly higher in P109 treated enclosures (personal observation). Such grazing has been noted in other studies (Lawrence et al., 2002). Invertebrate grazing on river biofilms grown in rotating annular bioreactors in the laboratory, for example, resulted in a significant reduction in both autotrophic biomass and exopolysaccharide (EPS) (Lawrence et al., 2002). Large snail

populations, however, were not observed in the saline ponds. The lack of PP stimulation in the saline ponds may therefore have been due to nutrient limitation (Waiser and Robarts, 1997) or other factors not monitored in this study. Further research, to test protein-to-carbohydrate and nutrient ratios within the biofilm community, however, is required to support this suggestion.

Although BP was not affected by the herbicide treatment, bacterial composition as measured by carbon utilization patters indicated changes. BIOLOG profiles from the present study suggest that there were differences in substrate utilization between control and treatment in all ponds. In addition, it was also found that the number of carbon sources with different utilizations between control and treatment were highest in P109 followed by P02. The number of carbon sources with different utilization patterns was lowest in P50 and P67. Measuring carbon utilization patterns using BIOLOG plates has been used previously (Lawrence et al., 2004, 2005; Ratcliff et al., 2006) and can be a sensitive indicator of community function and the impact of specific stresses (Konopka et al., 1998; Lawrence et al., 2004). Changes in substrate utilization may in turn alter symbiotic associations of bacteria to compensate for the lost or newly acquired function. In spite of this evidence, it is important to note that the BIOLOG profiles are developed from the culturable bacterial community as well as that this technique measures only the fastest growing portion of that culturable community (Bossio and Scow, 1998; Ratcliff et al., 2006).

In summary, the present study provided evidence that the herbicide mixture at maximum-exposure concentrations can exert effects on the microbial communities and such effects varied between ponds and may be dependent on the nutrient status of the aquatic system. Hormonal (stimulatory) effects of auxin-type herbicides on phytoplankton primary productivity were evident in the nutrient-sufficient freshwater pond (P109) while no such stimulatory effects were observed in the nutrient-deficient saline ponds (P02, P50 and P67). Nutrient status and salinity of

the aquatic systems appear may play an important role in the toxicity of herbicide mixture to microbial communities in prairie wetlands. Further research is required to evaluate the effects of these water quality parameters on herbicide toxicity.

In spite of high variability, results from this study using an *in situ* enclosure approach are perhaps more realistic than those derived in laboratory experiments. Herbicide mixtures are detected in various prairie aquatic ecosystems and it is important to investigate the effects of those mixtures. Although it is practically impossible to investigate effects of all mixture combinations; efforts, like the present study need to be undertaken in order to further understand effects of those mixture combinations currently found in these aquatic ecosystems.

4.5 Further Research

The following chapter (CHAPTER 5) is written in the form of manuscript and is accepted for for publication in Science of the Total Environment journal. This study investigated the effects of a herbicide mixture on microbial communities in two prairie wetlands. The herbicides in the mixture and their concentration used in this study were similar to those used in enclosure study (CHAPTER 4); however, this study incorporated more complexity by using whole-wetland land approach compared to mesocosms (CHAPTER 3) and enclosures (CHAPTER 4).

5. EFFECTS OF HERBICIDE MIXTURE ON MICROBIAL COMMUNITIES IN PRAIRIE WETLAND ECOSYSTEMS: A WHOLE WETLAND APPROACH[2]

Abstract

Wetlands in the prairie pothole region of Saskatchewan and Manitoba serve an important role in providing wildlife habitat, water storage and water filtration. These wetlands are regularly interspersed among agricultural operations where multiple pesticides are commonly used. Although mixtures of pesticides are often detected in these important aquatic ecosystems, very little information is known, regarding their effects. In this study, a curtained wetland approach was used to investigate effects of a herbicide mixture (2,4-D, MCPA, clopyralid, dicamba, dichlorprop, mecoprop, bromoxynil, and glyphosate) on the structure and function of microbial communities in an ephemeral wetland and a semi-permanent wetland. In the two studied wetlands, located in Manitoba Zero Till Research Association Farm, Brandon, Manitoba, herbicide treatment based on maximum-exposure scenarios had a significant effect on pelagic and biofilm phytoplankton productivity over relatively short time periods. The stimulation of phytoplankton productivity in the ephemeral wetland appeared to be the result of a hormonal effect of the auxin-type herbicides present in the mixture, similar to naturally occurring auxins. Herbicidal effects of auxin-type herbicides were also noticed in the semi-permanent wetland where phytoplankton productivity was suppressed during the first week as a result of concentration addition effect of the auxin-type herbicides present in the mixture. BIOLOG and pigment profiles of biofilm community suggested a change in the community structure in both

[2] A version of this chapter has been accepted for publication: Sura S., Waiser M.J., Tumber V.P., Farenhorst, A. (2012) Effects of herbicide mixture on microbial communities in prairie wetland ecosystems: A whole wetland approach. Science of the Total Environment.

wetlands. The changes in the microbial communities appeared to have affected invertebrate populations suggesting an inter-trophic disturbance.

5.1 Introduction

Wetlands function as ecotones, transitions between uplands and open water and therefore have characteristics of both aquatic and terrestrial ecosystems. These transition zones provide habitat for living organisms from both the land and water making them highly productive environments. The Prairie pothole region (PPR) across south-central Canada and the north-central United States contains more than four million wetlands which collectively cover 15 to 25 % of the region (Mitsch and Gosselink, 2000). The total wetland area in North America, however, has declined substantially, mostly due to human activities such as drainage (Dahl, 2000).

Prairie wetlands serve an important role in providing water storage, water filtration and wildlife habitat. Prairie wetland food webs consist of primary producers (free-living and attached algae, submerged and emergent plants), bacteria, benthic invertebrates, emergent insects and higher trophic level mammals and migrating waterfowl. These aquatic ecosystems are key ecological features of the prairie region, supporting 50 to 80 % of the North American waterfowl population and 50 % of other migratory birds each year (Mitsch and Gosselink, 2000; van der Valk, 1989).

One of the key geographical features of the PPR is that the wetlands are interspersed among agricultural fields where pesticides are commonly used (Donald et al., 1999; Waiser and Robarts, 1997). These pesticides can reach wetlands via spray drift, aerial deposition, surface runoff, or ground water flow (Grover et al., 1988; Waite et al., 1992) and pesticides are frequently detected in prairie wetlands (Donald et al., 1999, 2001, 2007; Waite et al., 2004). Seven herbicides most commonly found in prairie waters (drinking water reservoirs, wetlands and farm dugouts) are: 2,4-D [2-(2,4-dichlorophenoxy)acetic acid], MCPA [2-(4-chloro-2-methylphenoxy)acetic acid], dicamba [3,6-dichloro-2-methoxybenzoic acid], clopyralid [3,6-dichloropyridine-2-carboxylic

acid], dichlorprop [2-(2,4-dichlorophenoxy)propanoic acid], mecoprop [2-(4-chloro-2-methylphenoxy)propanoic acid], and bromoxynil [3,5-dibromo-4-hydroxybenzonitrile] (Donald et al., 2007). These herbicides are also among the most widely used in PPR for crop production (Waiser and Holm, 2005). There is limited information regarding the environmental fate (Degenhardt et al., 2011) and wildlife impacts of these herbicide mixtures in prairie wetlands, and how their fates and impacts vary with wetland hydrology.

Microbial communities consisting of phytoplankton and bacteria are vital members of the wetland food web, where they play significant roles in carbon, nitrogen and phosphorus cycling, as well as degradation of pesticides and decomposition of organic matter (DeLorenzo et al., 2001; Waiser and Robarts, 2004). Any stressor (such as a pesticide) which might affect phytoplankton and bacterial productivity may resonate to higher trophic levels; for example, zooplankton which feed on algae, and ducks which feed on zooplankton and benthic invertebrates (Waiser and Holm, 2005). These effects are likely more prevalent when pesticides having different mode of actions are present because there is a greater potential for the chemicals to affect multiple components of the ecosystem.

In this study, a whole-wetland approach was used to investigate effects of a mixture of eight herbicides on the productivity and biomass of pelagic and attached wetland microbial communities (algae, bacteria) in an ephemeral wetland as well as a semi-permanent wetland. The mixture included 2,4-D, MCPA, dicamba, clopyralid, dichlorprop, mecoprop, bromoxynil, and glyphosate [2-(phosphonomethylamino)acetic acid]. Glyphosate was added because it is one of the most commonly and heavily used herbicides in Canada (Ribo, 1986) and has been detected in prairie wetlands (Messing et al., 2011).

The intent of the current study was to provide much-needed knowledge regarding effects of this environmentally relevant herbicide mixture on sensitive prairie wetland microbial communities. Ecosystem-scale experiments like whole-wetland studies, whether replicated or not, are important steps in understanding effects of anthropogenic substances, like herbicides, on ecological processes in aquatic ecosystems. Information generated from such studies will assist regulatory agencies in making informed decisions in estimating risks due to pesticides and developing strategies to mitigate adverse effects of pesticides in these ecologically important ecosystems (Carpenter, 1996; Schindler, 1998).

5.2 Materials and Methods

5.2.1 Study Site and Design

Two wetlands, located on the Manitoba Zero Till Research Association Farm (49^{o}53' N 99^{o}58' W), 20 km north of Brandon, in the PPR of Manitoba, Canada were selected as study sites. Pond 227, a smaller ephemeral wetland (Class 3 pothole) (hereafter referred to as wetland E) has a shallow central area with two deeper zones on either side (giving an illusion of two separate wetlands) (Figure 5.1). Wetland E is characterized by a marshy riparian zone covered with tall rush (*Scirpus* sp.) and cattail (*Typha* spp.). Pond 158, the bigger, semi-permanent wetland (Class 4 pothole) (hereafter referred to as wetland SP) has a riparian zone covered with cattail, reed (*Phragmites* spp.), and sedge (*Carex* spp.) (Figure 5.2).

Each wetland was divided into two halves ('control' and 'treated') using laminated polyvinyl curtains. Curtains were anchored on each side of the wetlands using nylon ropes threaded though grommets on the top of the curtains and then the ropes strapped around iron posts driven into the ground. Heavy metal chains threaded through a pouch along the bottom of the curtains ensured that the curtain remained embedded in the sediments. Finally, to ensure maintenance of a leak-proof barrier, sand bags were placed on top of the chain on the control half of the wetland.

Curtains were installed in each wetland in early May 2008 and wetlands allowed to acclimatize *in situ* for 4 to 5 weeks before the start of the experiment.

Wetland water volumes were calculated using a digital elevation model (DEM) of the site based on wetland bathymetry, topography of the surrounding area as well as water depths in the wetland. Water depths were measured and volumes were calculated for both the wetland-halves prior to treatment. Details of water volumes can be found in Degenhardt et al. (2011).

5.2.2 Herbicide Treatment

A mixture of eight herbicides (commercially formulated) was used (Table 5.1) with the target concentration for each being the expected environmental concentration (EEC). The eight herbicides were mixed into approximately 30 L of water in a hand-operated sprayer equipped with a 1.5-m wand and then injected below the water surface. For wetland E which is a shallow pond, the herbicide mixture solution was applied by wading into the wetland and moving in a zigzag pattern to ensure homogeneity of the herbicide treatment. For wetland SP, an inflatable raft was used to inject herbicide mixture solution beneath the water surface upto a depth of 1.5 m. The raft was pulled in a zigzag pattern over the width and length of the wetland using ropes on either ends to ensure a homogenous application of herbicide mixture solution. EEC is the calculated concentration of a herbicide, in various environmental compartments, (e.g. water) based on maximum-exposure scenarios. In this study, EEC was based on direct overspray of herbicide at the recommended field application rate on a 0.5-m deep water body (Cessna et al., 2006). The resulting concentration became the target concentration for each herbicide in the mixture. These target concentrations are approximately 10 times (for dicamba) to 50 times (for MCPA) greater than their respective herbicide Canadian Water Quality Guidelines for the Protection of Aquatic Life in Freshwater (Table 5.1). Both wetland target herbicide concentrations were intended to be same. However, due to an overestimation of the volume of

wetland SP that was not discovered until after herbicide treatment, the resulting target concentration of each herbicide for this wetland was 28 % greater than that for wetland E (Degenhardt et al., 2011). Treatments at high herbicide concentrations such as these provide experimental evidence for worst case scenarios as well as information on herbicide fate.

A conservative tracer (bromide ion) was added to the treated half in both the wetlands to confirm no cross contamination from the treated to the control half of the wetlands. Details on the application of the herbicides, the conservative tracer, and the detected herbicide concentrations can be found in Degenhardt et al. (2011, 2012).

Figure 5.1 Photograph showing smaller ephemeral wetland (wetland E) and the curtain installed separating ‘control’ and ‘treatment’ halves at Manitoba Zero Till Research Association Farm near Brandon, Manitoba, Canada.

Figure 5.2 Photograph showing bigger semi-permanent wetland (wetland SP) and the curtain installed separating ‘control’ and ‘treatment’ halves at Manitoba Zero Till Research Association Farm near Brandon, Manitoba, Canada.

Table 5.1 List of herbicides used, their trade names, active ingredients, concentrations, recommended application rates, and wetland fortification target concentrations.

Herbicide	Trade name	Herbicide formulation	Concentration of active ingredient (A.I.) (acid equivalent) (g/L)	Recommended application rates for cropland (g/ha)[a]	Target concentration in wetland E (A.I.) (μg/L)	Target concentration in wetland SP (A.I) (μg/L)	Guideline value[b] (μg/L)
2,4-D	Nufarm Estaprop PLUS	2-ethylhexyl ester	282	495	100	128	4
MCPA	Nufarm MCPA Amine 500	Dimethylamine salt	500	625	127	162	2.6
Clopyralid	Lontrel 360	Monoethanolamine salt	360	266	53	68	NA[c]
Dicamba	Oracle	Dimethylamine salt	480	468	95	121	10
Bromoxynil	Pardner	Octanoate / heptanoate ester	280	308	62	80	5
Dichlorprop	Nufarm Estaprop PLUS	2-ethylhexyl ester	300	525	106	136	4
Mecoprop	Mecoprop	Potassium salt	150	895	181	230	4
Glyphosate	Glyphos	Isopropylamine salt	360	360	72	92	65

[a] These application rates represent recommended maximum safe rates for wheat and barley crops in Saskatchewan and Manitoba (Saskatchewan Ministry of Agriculture, 2008).
[b] Water Quality Guideline for the Protection of Aquatic Life in Freshwater (CCME, 1999).
[c] NA = Not available.

5.2.3 Sampling and Water Analysis

Surface water temperature, pH, dissolved oxygen (DO), alkalinity and specific conductivity were measured between 10:00 and 11:00 h on each sampling date using a YSI 650MDS data display and logging unit connected to a 600XLM-0 multi-parameter water quality monitoring probe (YSI Inc., Ohio, USA). Every two weeks, composite water samples were collected from control and treated side of each wetland for nutrient analyses including total phosphorus (TP), ammonium nitrogen (NH_4^+), and dissolved organic carbon (DOC). Samples were prepared according to established methods (EC, 1992), placed on ice, and transported to the laboratory for analysis. NH_4^+ and TP were analyzed using a Seal Colorimeter AA-3 (Seal Analytical, Norderstedt, Germany). DOC was analysed using DOC analyzer (Tekmar-Dohrmann Phoenix 8000, Ohio, USA). Calcium (Ca), magnesium (Mg), sodium (Na), and potassium (K) were also measured using ion chromatography (ICS-1000, Dionex Canada Ltd., ON, Canada) (EC, 1992).

For the pelagic community sampling, water samples from both wetlands were collected on Days -4 (pre-treatment), 1, 2, 3, 7, 14, 21, and 28 (post-treatment) into clean 2-L amber PVC bottles using a polyethylene tube (inner diameter of 4 cm) connected to a variable speed peristaltic sampling pump (Portable Masterflex Sampling Pump-7570, Cole-Parmer Instrument Company, Chicago, USA) at a rate of 300 to 400 mL/min. A large plastic funnel equipped with a 150-μm Nitex mesh screen (Dynamic Aqua-Supply Ltd., BC, Canada) was used to remove large zooplankton from the collected water. The screened water was subsequently used to determine primary productivity (PP), chlorophyll *a* content (Chl *a*), bacterial productivity (BP), bacterial numbers (BN). The sample replicates collected from each side of the wetland could represent pseudoreplication; however, it is practically impossible to find two or more ponds with similar biotic and abiotic characteristics.

For the biofilm community sampling, attached communities (biofilms) were grown *in situ* on 2.54-cm^2 diameter glass coverslips. Prior to deployment, coverslips were loaded onto PVC plates, previously etched with tracks to hold the round coverslips. The plates were then fitted onto a floating plastic-platform. Platforms were deployed into control and treated halves of the each wetland one day prior to treatment. Four float balls mounted on four corners of the platform kept coverslips at a constant depth of approximately 20 cm below water surface. Platforms were tied to a post inserted into the bottom sediments to maintain position. Coverslips with attached biofilms were harvested after 7, 14, and 21 days of growth, post-treatment. These biofilms were used to measure PP, BP, and Chl *a* content.

5.2.4 Pelagic Community Analysis

5.2.4.1 Measurement of primary productivity (PP)

PP was determined using a standard light/dark bottle ^{14}C method which measures the ability of phytoplankton to take up and incorporate tracer amounts of radioactive isotopes into organic matter during photosynthesis (Wetzel and Likens, 1991). Volumetric rates of PP (mg C m^{-3} h^{-1}) were calculated from ^{14}C incorporation rates and ^{12}C concentrations (using temperature, pH and alkalinity data) available to phytoplankton (Robarts et al., 1992). Daily rates were estimated by multiplying hourly rates by 10 (Cole et al., 1988).

Alkalinity of water was determined by end point titration with 0.01N H_2SO_4 solution (Clesceri et al., 1998) using a TitraLab TIM850 titration system with SAC80 autosampler (Radiometer Analytical SAS, France) linked to TitraMaster 85 software.

5.2.4.2 Measurement of bacterial productivity (BP)

BP was determined by rate of incorporation of a radioactively-labelled nucleotide (^3H-thymidine) into nucleic acids (Robarts and Wicks, 1989). BP rates were calculated from ^3H-thymidine incorporation using a conversion factor for a eutrophic lake of 2.0 x 10^{18} bacterial

cells produced per mole ^{3}H-thymidine (Bell et al., 1983; Coveney and Wetzel, 1988). A factor of 20 fg C per bacterial cell was then used to convert cell numbers to an estimate of carbon produced (Lee and Fuhrman, 1987; Reitner et al., 1999). Because the carbon content of most bacterial cells is in the 10 to 20 fg C /cell range (Cotner and Biddanda, 2002), the upper limit was chosen to represent carbon content of bacterial cells in eutrophic ecosystems. Daily volumetric rates were estimated by multiplying hourly rates by 24 (Cole et al., 1988).

5.2.4.3 Estimation of bacterial numbers

The DAPI (4,6-diamidino-2-phenylindole) fluorescent DNA staining method was used for bacterial enumeration (Porter and Feig, 1980). Four replicate 10-mL aliquots of screened water from each side of the wetland were pipetted into sterile Vacutainer tubes and preserved with 200 µL of Lugol's solution. Samples were stored at 4 ^{o}C until further analysis. Subsequently, bacteria were stained with DAPI and counted using epifluorescence microscopy (Waiser, 2001a). At least 200 cells were counted for each replicate.

5.2.4.4 Estimation of phytoplankton biomass

Phytoplanktonic biomass was estimated as Chl *a* (Wetzel and Likens, 1991). Water samples were filtered through 47-mm Whatman GF/C filters (nominal pore size 1.2-µm). Chl *a* was extracted using a boiling ethanol technique and subsequently analysed fluorometrically using a Turner Design Model 10-AU digital fluorometer (Turner Designs, Sunnyvale, CA) (Waiser and Robarts, 1997).

5.2.5 Biofilm Community Analysis

5.2.5.1 Measurement of primary productivity (PP)

For biofilm primary productivity, coverslips were randomly harvested and placed in crystallization dishes containing 20 mL of 0.2-µm filter sterilized water and 450 µL of $NaH^{14}CO_3$. Dark control dishes were covered with foil and all were incubated for one hour.

Coverslips were then removed, individually placed in 50 mL Falcon tubes containing 50 mL 0.2-μm filter sterilized water, placed on ice and transported to the laboratory (Waiser, 2001b). Coverslips were crushed using a clean glass stirring rod, and contents subsequently filtered through 47-mm 0.45-μm pore-size Whatman cellulose nitrate filters under gentle vacuum. Filters were then treated and counted as noted above for pelagic PP. Biofilm PP rates were calculated as described above for pelagic samples except that rates were based on the area of the coverslip, not the volume filtered.

5.2.5.2 Measurement of bacterial productivity (BP)

The setup for BP incubation was similar to PP except that the incubation was carried out in 50-mL Falcon tubes instead of crystallization dishes. At each sampling time (7, 14, and 21 days), coverslips were harvested randomly and each placed into a Falcon tube containing 20 mL filter sterilized water and 334 μL methyl [^{3}H]thymidine solution (20 nM) (Perkin Elmer). Control tubes received 2 mL formalin. Tubes were incubated *in situ* for 1 h. Thymidine incorporation was stopped by adding 2 mL of 5N NaOH followed by 2 mL formalin. DNA was extracted in the laboratory as described above in pelagic BP except that 2 mL of 100 % TCA was added to each sample. BP rates were calculated as for pelagic samples except that the coverslip area was used instead of volume filtered (Waiser, 2001b).

5.2.5.3 Estimation of phytoplankton biomass

Four coverslips harvested from each side of the wetland were each placed in 50-mL centrifuge tubes containing 10 mL of 90 % ethanol. Tubes were placed on ice and transported back to laboratory. All coverslips were then crushed, samples filtered and Chl *a* extracted and measured as described above in pelagic section.

5.2.5.4 Bacterial community structure analysis

Assays of bacterial community structure were conducted using BIOLOG EcoPlates, a technique for testing bacterial carbon source utilization from naturally occurring communities (Garland and Mills, 1991). For these analyses, one coverslip from each of treated and control sides was placed in a 50 mL centrifuge tube containing 5 mL of 0.2-μm filter sterilized water. Samples were stored on ice and transported to the laboratory. Coverslips were crushed using a clean glass rod, contents vortexed and then centrifuged at 3000 g for 5 min. 100 μL of the supernatant was pipetted into each well of a 96 well BIOLOG EcoPlate (BIOLOG Inc., Hayward, CA, USA). One BIOLOG EcoPlate was used for each side of the wetland at each of the 3 sampling times. Inoculated plates were incubated at 22 ^{o}C and absorbance read after 7 days on an Emax BIOLOG Microstation plate reader (Molecular Devices, Inc., Sunnyvale, CA, USA) at 590 nm.

5.2.5.5 Pigment profile analysis

One coverslip from each side of the wetland was placed into a 50-mL Falcon tube containing 20 mL of filter sterilized wetland water. Tubes were transported to the laboratory on ice and stored at – 80 ^{o}C until further analysis. Prior to analysis, samples were thawed, coverslips crushed and contents filtered through a 25-mm pre-combusted GF/C filter in a dark room. Filters were stored separately in 2-mL Eppendorf Snap-Cap microcentrifuge tubes (Fisher Scientific, Ottawa, Canada) at – 80 ^{o}C until further analysis. Pigments were subsequently extracted and analyzed by high performance (pressure) liquid chromatography (HPLC) (Leavitt and Hodgson, 2001). Relative concentrations of all pigments were obtained by normalizing all values to Chl *a*. These data transformation allowed comparisons between control and treatments.

5.2.6 Statistical Analysis

A student's *t*-test was conducted on each water quality parameter to detect any significant differences between control and treatment of each wetland during the study period. A two-way repeated measures analysis of variance (RM ANOVA) was conducted on each response variable (PP and BP rates, and Chl *a* content) from pelagic communities over time to detect any significant differences between control and treatment. In addition, a student's t-test was performed on each response variable on Day 28 (last sampling day) from the pelagic communities to detect any significant differences between control and treatment at the end of the study period.

A student's *t*-test was performed on each response variable (PP and BP rates, and Chl *a* content) from biofilm communities on each sampling day (Days 7, 14 and 21) to detect any significantly differences between control and treatment on the respective days. A student's *t*-test was used for biofilm data because samples from each sampling day were independent. A one-way ANOVA was performed on BIOLOG data for each of 31 carbon substrates in BIOLOG EcoPlate to detect differences between control and treatment. The level of significance was $p < 0.05$. All statistical tests were conducted using SAS statistical software package, version 9.1 (SAS Institute Inc., Cary, North Carolina, USA).

5.3 Results

5.3.1 Water Quality Parameters

Temperature, dissolved oxygen (DO), pH, alkalinity, dissolved organic carbon (DOC) NH4+, total phosphorus (TP), and K^+, measured weekly, were found to be similar on control and treated sides of both E and SP wetlands (Table 5.2). Specific conductance, Na^+, Mg^{+2}, and Ca^{+2}, measured weekly, were similar on control and treated sides of the wetland SP, while they were

on average higher on the treated side of wetland E as compared to the control (*t*-test, $p<0.05$, n=7).

Table 5.2 Physical and chemical water quality parameters on each side of ephemeral (E) and semi-permanent (SP) wetlands (values are averages of measurements on various (Days 1, 2, 3, 7, 14, 21, and 28) sampling days ± standard deviations, n=7). Significantly different values are indicated in bold.

Parameter	**Ephemeral Wetland**		**Semi-Permanent Wetland**	
	Control	**Treated**	**Control**	**Treated**
Temperature (^{o}C)	13 ± 3	13 ± 2	17 ± 3	17 ± 3
Specific Conductance (μS/cm)	**530 ± 70**	**820 ± 60**	1360 ± 240	1240 ± 100
Dissolved oxygen (DO) (mg/L)	5.0 ± 2.8	4.2 ± 2.3	9.3 ± 1.4	8.9 ± 1.6
pH	7.2 ± 0.3	7.2 ± 0.2	8.6 ± 1.1	8.9 ± 1.6
Alkalinity (mg/L as $CaCO_3$)	215 ± 46	233 ± 74	258 ± 42	241 ± 41
Dissolved organic carbon (mg/L)	39 ± 4	42 ± 7	24 ± 3	25 ± 2
NH_4^+ (mg/L)	<0.6	<0.6	<0.6	<0.6
Total Phosphorus (TP) (mg/L)	1.6 ± 0.6	2.2 ± 0.6	0.8 ± 0.1	1.1 ± 0.4
Ca^{+2} (mg/L)	**57 ± 6.2**	**85 ± 8.5**	113 ± 15	105 ± 14
Mg^{+2} (mg/L)	**26 ± 3.5**	**51 ± 5.8**	157 ± 33	136 ± 25
Na^+ (mg/L)	**12 ± 1**	**24 ± 2**	55 ± 12	51 ± 10
K^+ (mg/L)	37 ± 9	33 ± 9	28 ± 5	33 ± 5

5.3.2 Pelagic Communities

5.3.2.1 Ephemeral (E) wetland

Pelagic PP rates were significantly different over time when treated and control halves were compared (RM ANOVA, $p < 0.05$) (Figure 5.3A). Although pelagic PP rates in both control and treated halves of the wetland were similar on Day -4 and Day 1, rates steadily increased in the treated half throughout the study period while, in the control, rates were consistently low except for a strong increase in pelagic PP rates observed on Day 28. In fact, by Day 28 (1201 ± 194 mg carbon/m^3/day), rates in the control were significantly higher than those in the treated half (587 ± 38 mg carbon/m3/day) (*t*-test, $p < 0.05$). A similar trend was seen in algal biomass with pelagic Chl *a* with Chl *a* increasing gradually in the treated half over the study period while such an increase was not found in the control half until the last sampling day when Chl *a* was significantly higher than treatment (*t*-test, $p < 0.05$) (Figure 5.3A).

Pelagic BP rates were significantly different over time for treated and control halves (RM ANOVA, $p < 0.05$) with BP rates over the study period trending lower in the treated half than in the control (Figure 5.3B).

5.3.2.2 Semi-permanent (SP) wetland

Pelagic PP rates were significantly different over time when treated and control halves were compared (RM ANOVA, $p < 0.05$) (Figure 5.4A). Although rates on day -4 (pre-treatment) were similar on both sides of the wetland, rates increased more strongly in the control side than the treated half. PP rates in the treated side were significantly lower than those on control side until Day 7 (*t*-test, $p < 0.05$). Although PP on treated side increased after Day 7, rates were generally still lower than the control side until study end.

Chl *a* concentrations followed a similar pattern as PP with Chl *a* being 390 % (control half) and 160 % (treated half) greater on Day 28 compared to pre-treatment levels (Figure 5.4A).

Although Chl *a* was significantly higher (*t*-test, $p < 0.05$) in the treatment than control side before herbicide additions, Chl *a* was significantly lower in the treatment than control side on day 28 (*t*-test, $p < 0.05$).

Pelagic BP rates were significantly different over time for treated and control halves (RM ANOVA, $p < 0.05$) (Figure 5.4B) with average BP rates in the treatment half being lower than the control on Days 3 and 7 but higher on Days 2, 14, 21 and 28.

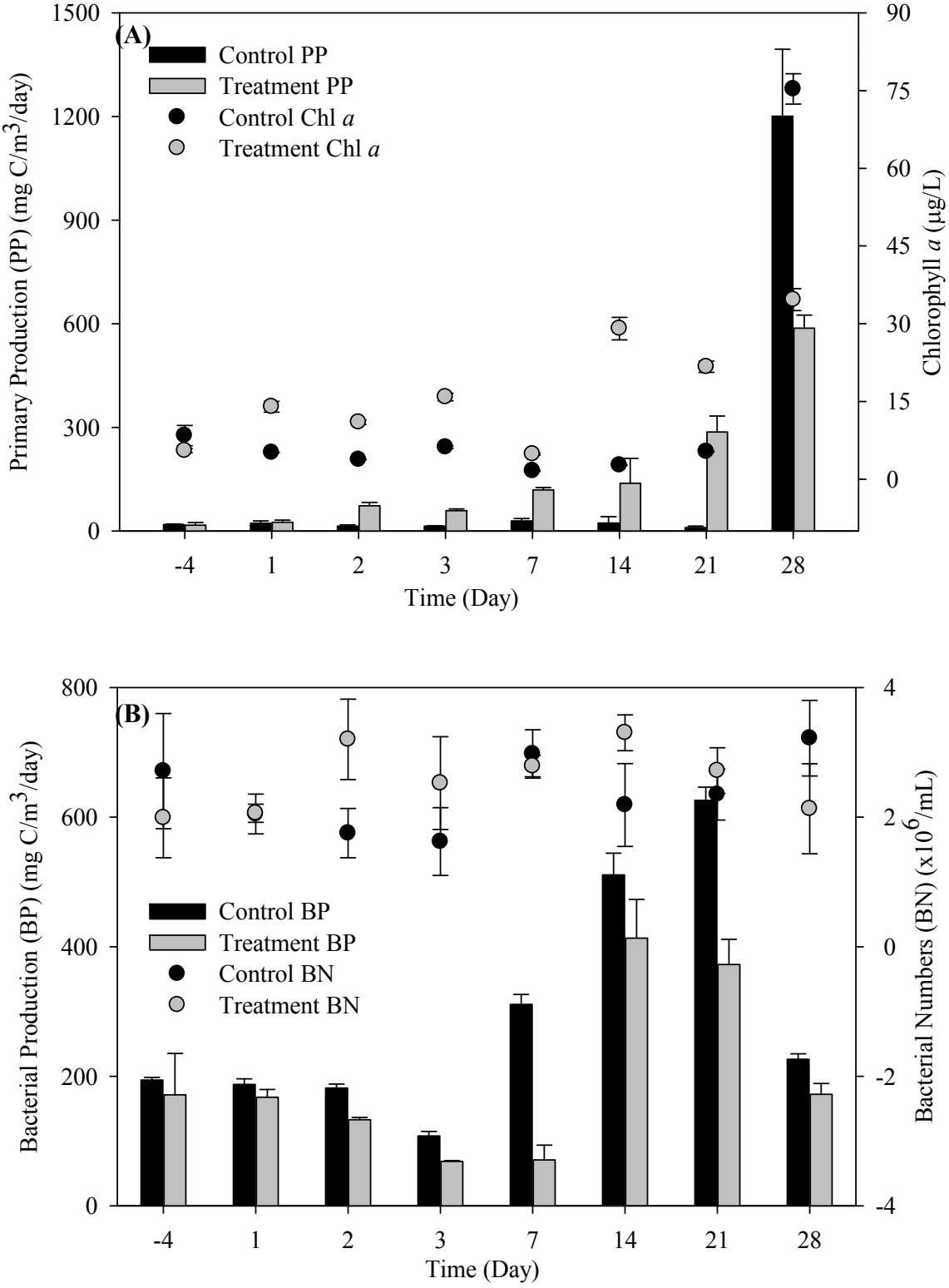


Figure 5.3 Pelagic primary productivity (PP) rates and chlorophyll *a* (Chl *a*) (A) and pelagic bacterial productivity (BP) rates and bacterial numbers (BN)) (B) in wetland E over the 28 day study period. Error bars represent SD, n = 4.

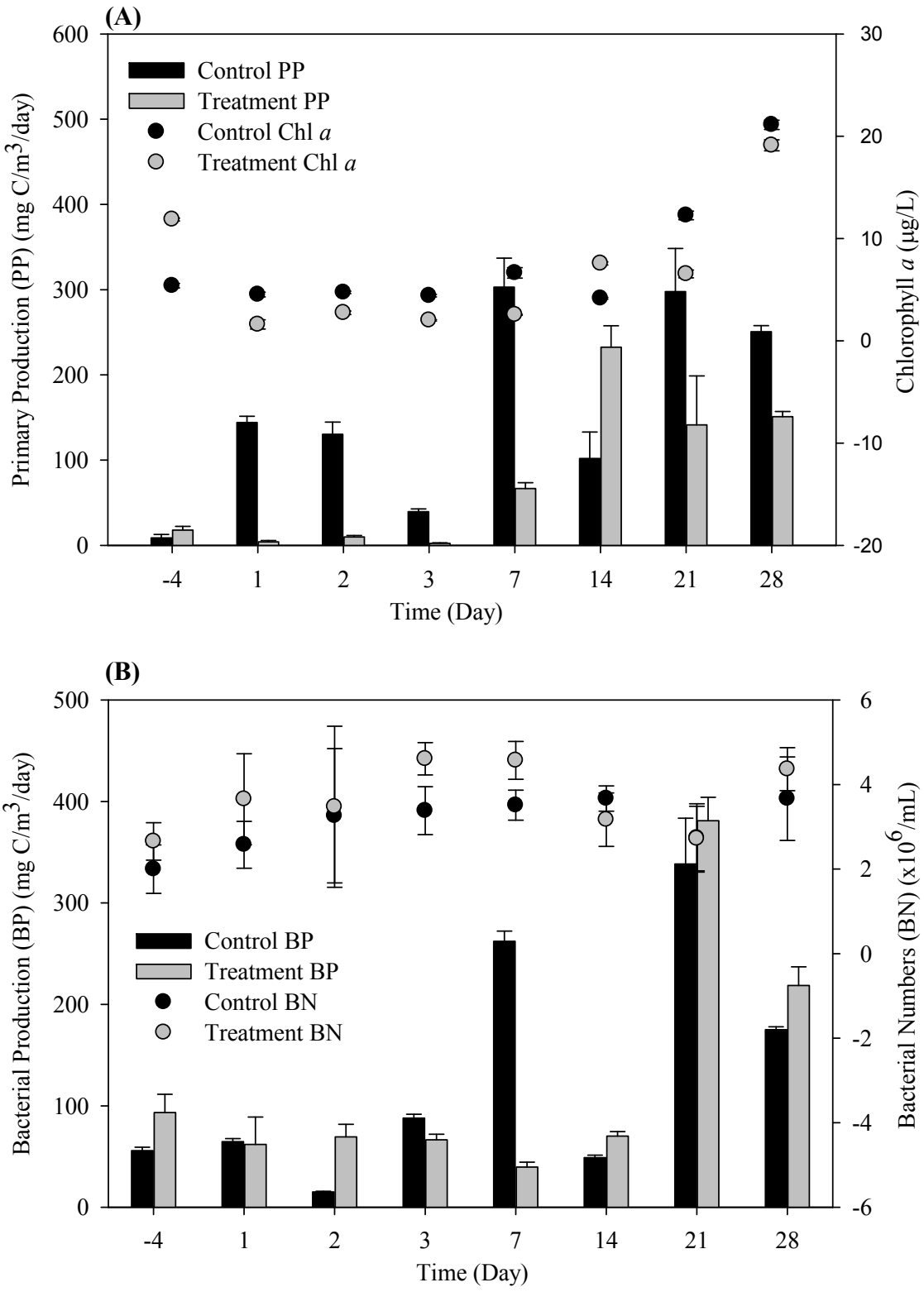


Figure 5.4 Pelagic primary productivity (PP) rates and chlorophyll *a* (Chl *a*) (A) and pelagic bacterial productivity (BP) rates and bacterial numbers (BN) (B) in wetland SP over the 28 day study period. Error bars represent SD, n=4.

5.3.3 Biofilm Communities

5.3.3.1 Ephemeral (E) wetland

Biofilm PP rates in wetland E were significantly higher in the treated half than the control on Days 7 and 21 (*t*-test, $p < 0.05$), while no significant difference was detected on Day 14 (Figure 5.5A). PP rates in treatment side of wetland E, however, decreased significantly on Day 14 and Day 21 compared to Day 7 (*t*-test, $p < 0.05$), while no significant changes in rates over three sampling days were observed in control side. Although biofilm Chl *a* concentrations on the treatment side were numerically higher on all three days (Days 7, 14 and 21) compared to control, differences were significant only on Day 14 (Figure 5.5A). Average BP rates were numerically lower on the treatment side than the control on all three sampling days, however, the difference was significant only on Days 7 and 21 (*t*-test, $p < 0.05$) (Figure 5.5B).

According to the BIOLOG results, there were differences in carbon utilization on all three sampling days (Table 5.3). The differences in carbon utilization between control and treated halves were low because there was no difference in utilization of the majority (26 to 28) of the 31 carbon compounds.

With respect to pigment profiles, Chl *c1* and Chl *c2* were not found on the treatment side but were present in the control biofilms on Days 14 and 21 (Figure 5.6). The pigment fucoxanthin increased in treatment on Day 14 and 21. As well, the pigments alloxanthin, diatoxanthin, lutein and zeaxanthin, Chl *b*, pheophytin *b* and pheophytin *a*, although lower in treatment compared to control on Day 7, all increased above control biofilms on Day 21.

5.3.3.2 Semi-permanent (SP) wetland

Although biofilm PP rates on the treated side of wetland SP were significantly higher on Day 7 (*t*-test, $p < 0.05$) no significant differences were observed on Days 14 and 21 (*t*-test, $p > 0.05$) (Figure 5.5A). PP rates on Day 14 in treatment side of wetland SP were significantly lower than

those on Days 7 and 21 (*t*-test, $p < 0.05$), while a progressive increase in PP rates was observed on the control side. Despite similar Chl *a* concentrations on treatment and control sides on Day 7, significantly higher biomass in treatment as compared to control was observed on Day 14 (*t*-test, $p < 0.05$) with the opposite noted on Day 21 (*t*-test, $p < 0.05$) (Figure 5.5A). Biofilm BP rates in wetland SP were significantly higher on treatment side than control on Days 7 and 21 (*t*-test, $p < 0.05$) (Figure 5.5B).

The BIOLOG data indicated that on Day 7 carbon utilization was significantly different in 10 of 31 carbon substrates, on Day 14 in 8 substrates and on Day 21, 5 substrates when control and treatment biofilms were compared (Table 5.3).

Pigment profiles indicated that Chl *c1*, Chl *c2*, and fucoxanthin concentrations from treated biofilms on Days 7 and 14 were less in the treatment than control, while on Day 21, Chl *c1* and Chl *c2* increased while fucoxanthin remained lower than control (Figure 5.7). Chl *b* in treated side biofilms was higher than control on all three sampling days. Other differences noted were that although pheophytin *a* and beta-carotene were not found in control biofilms on Day 7, they were present on Day 21 but at concentrations less than those from treated biofilms.

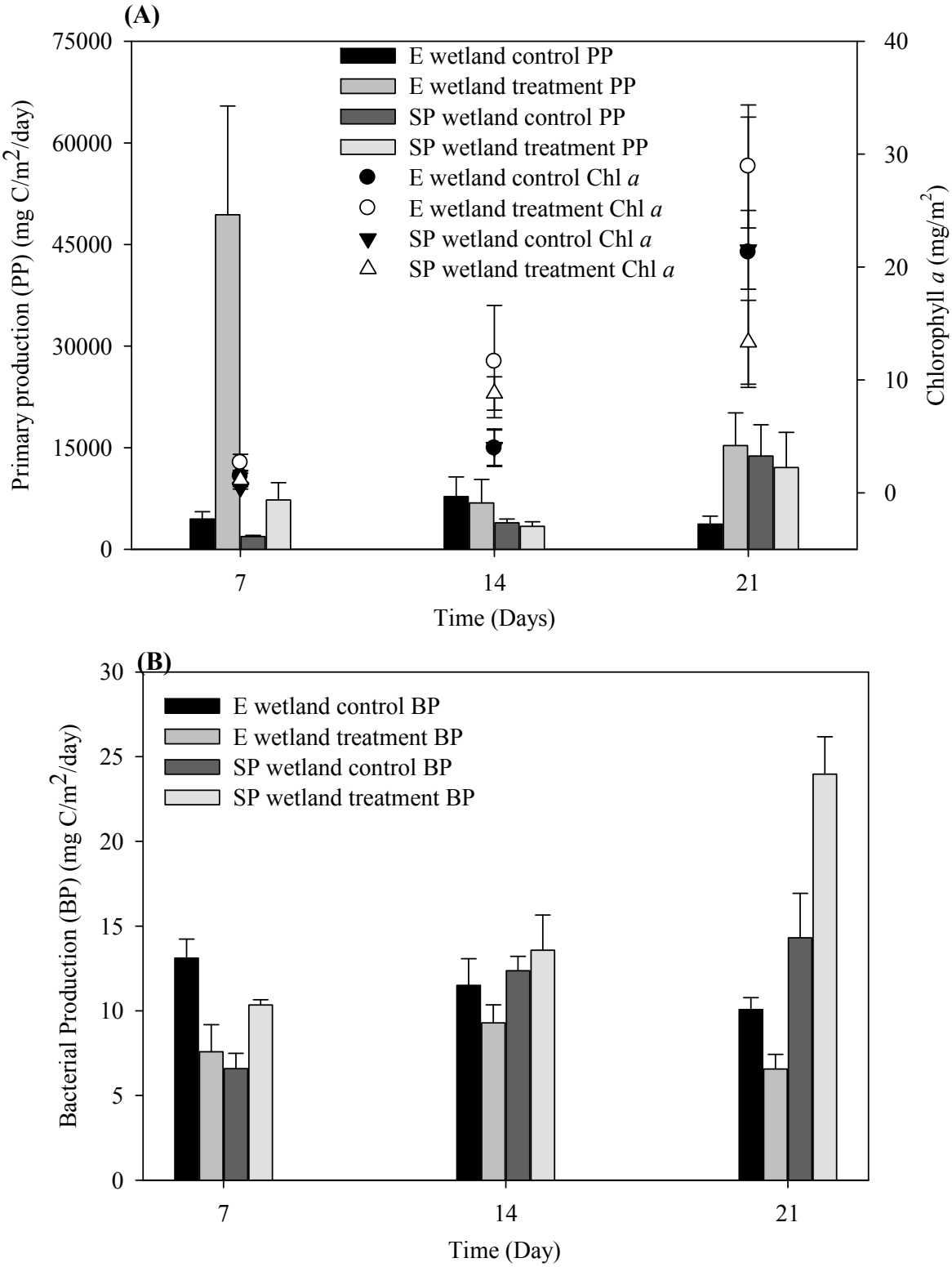


Figure 5.5 Biofilm primary productivity (PP) rates and chlorophyll *a* (Chl *a*) (A) and biofilm bacterial productivity (BP) rates (B) in both wetlands E and SP. Error bars represent SD, n=4.

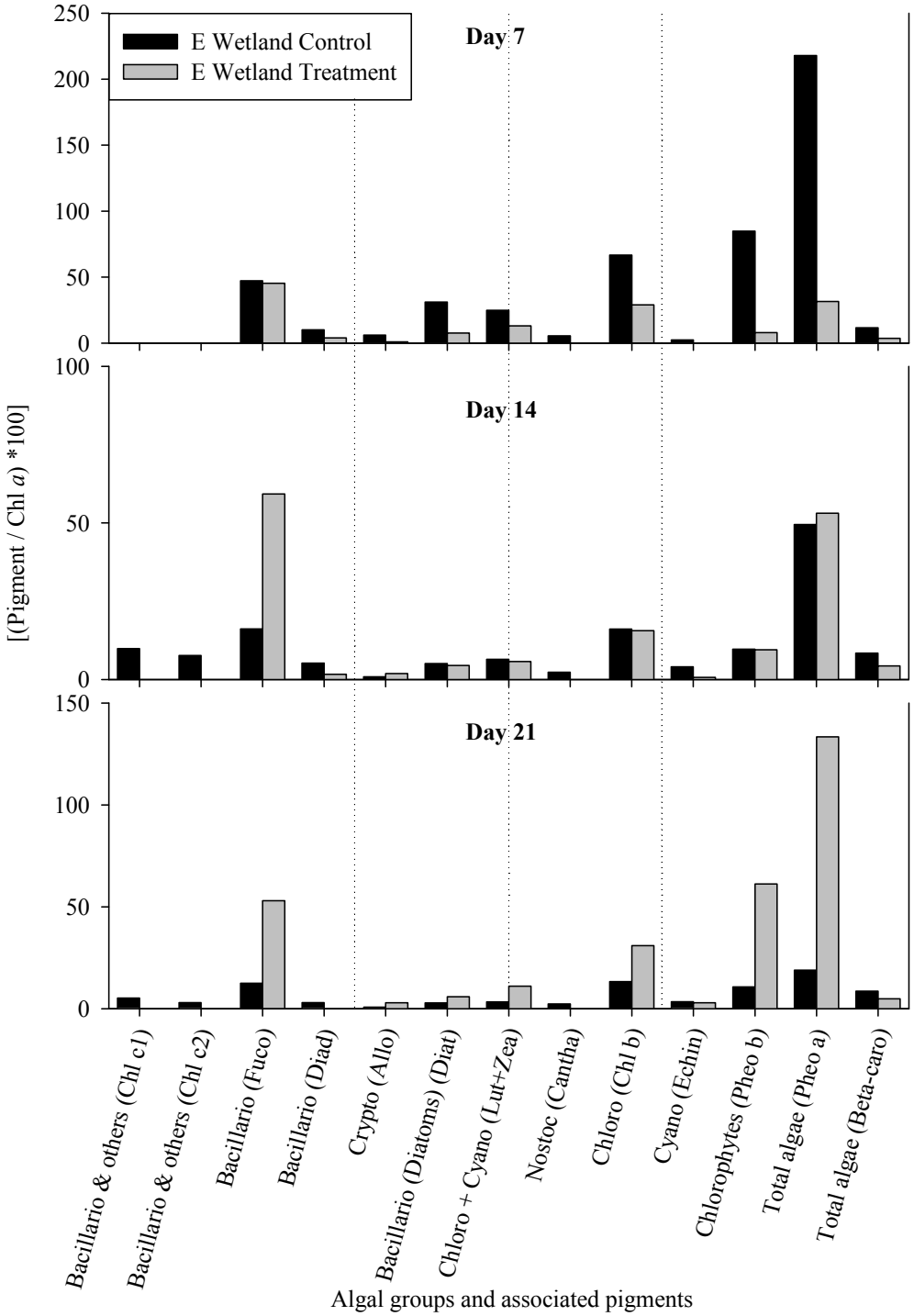


Figure 5.6 Pigment profiles from biofilms in wetland E on Days 7, 14, and 21 include compounds from Bacillariophyceae and Chrysophyceae (chlorophyll *c1*, chlorophyll *c2*, fucoxanthin, diadinoxanthin, diatoxanthin (mainly diatoms)), cryptophytes (alloxanthin), chlorophytes (lutein, chlorophyll *b*, pheophytin *b*), cyanobacteria (zeaxanthin, echinenone), Nostocales cyanobacteria (canthaxanthin), total algae (pheophytin *a*, β-carotene). Lutein and zeaxanthin are presented together as they may not be resolved into individual peaks on HPLC.

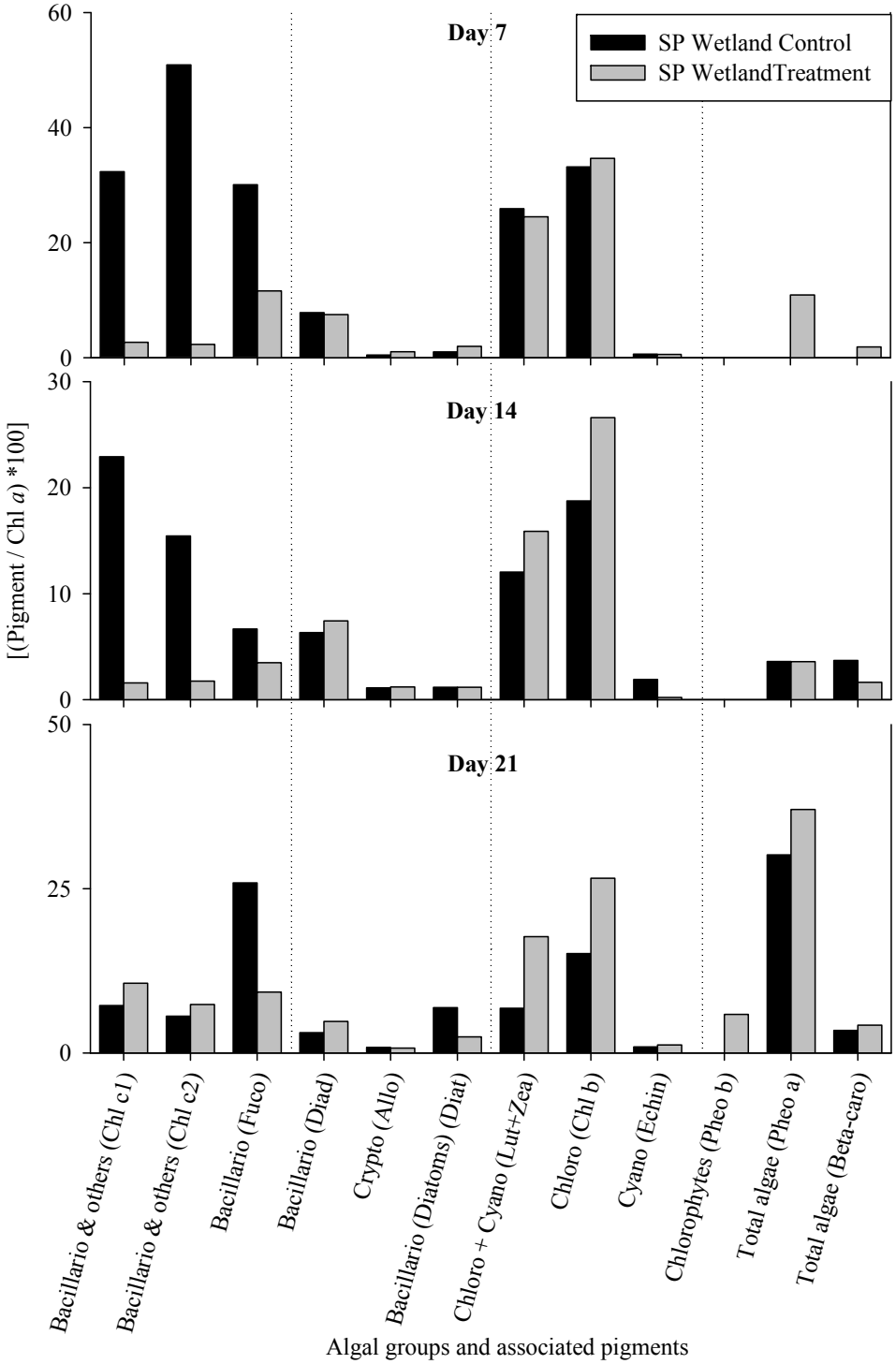


Figure 5.7 Pigment profiles from biofilms in wetland SP on Days 7, 14, and 21 include compounds from Bacillariophyceae and Chrysophyceae (chlorophyll *c1*, chlorophyll *c2*, fucoxanthin, diadinoxanthin, diatoxanthin (mainly diatoms)), cryptophytes (alloxanthin), chlorophytes (lutein, chlorophyll *b*, pheophytin *b*), cyanobacteria (zeaxanthin), total algae (pheophytin *a*, β-carotene). Lutein and zeaxanthin are presented together as they may not be resolved into individual peaks on HPLC.

Table 5.3 Summary statistics of BIOLOG plate incubations of biofilm samples collected from wetlands E and SP on Days 7, 14, and 21. D denotes significant difference between control (C) and Treatment (T) within wetland on the same day. ($p < 0.05$).

Carbon sources in the BIOLOG Eco-plate	Wetland E						Wetland SP					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	C	T	C	T	C	T	C	T	C	T	C	T
POLYMER												
α-Cyclodextrin					D	D						
Tween 40							D	D	D	D	D	D
Tween 80							D	D	D	D	D	D
CARBOHYDRATE												
D-Xylose	D	D	D	D								
i-Erythritol							D	D			D	D
Glycogen			D	D			D	D				
ß-Methyl-D-Glucoside												
N-Acetyl-D-Glucosamine							D	D				
D-Cellobiose									D	D		
α-D-Lactose											D	D
D-Mannitol	D	D	D	D	D	D	D	D	D	D		
CARBOXYLIC ACID												
2-Hydroxy Benzoic Acid			D	D					D	D	D	D
α-Ketobutyric Acid												
Itaconic acid												
D-Malic Acid												
D-Galactonic Acid γ-Lactone												
D-Glucosaminic Acid												
4-Hydroxy Benzoic Acid							D	D	D	D		
γ-Hydroxybutyric Acid							D	D	D	D		
D-Galacturonic Acid												
AMINO ACID												
L-Threonine												
Glycyl-L-Glutamic Acid			D	D								
L-Phenylalanine							D	D				
L-Serine												
L-Arginine												
L-Asparagine	D	D					D	D				
AMINE												
Putrescine												
Phenylethylamine												
PHOSPHORYLATED												
D,L-α-Glycerol Phosphate												
Glucose-1-Phosphate									D	D		
ESTER												
Pyruvic Acid Methyl Ester												

5.4 Discussion

5.4.1 Pelagic Communities

Based on the maximum-exposure scenario used in this study, the herbicide mixture had differing effects on pelagic PP and Chl *a* in the studied wetlands. While PP and Chl *a* were enhanced for at least 21 days post-treatment in wetland E, the same end points were suppressed for at least seven days post-treatment in wetland SP. One of the possible reasons for the observed differences between the wetlands may be linked to hydrology. As reported by Degenhardt et al., (2011), precipitation events in the first week post-treatment (90 mm in total) increased water volume by 330 % in wetland E but only by 30 % in wetland SP. Hence, herbicide concentrations were generally less in wetland E than SP. In addition, the total auxin-type herbicide mixture concentration was, for example, 527 μg/L on Day 1 and 121 μg/L on Day 28 in wetland E while in wetland SP, it was 792 μg/L on Day 1 and 282 μg/L on Day 28 (calculated from data in Degenhardt et al., (2011)). Hence, the total auxin-type herbicide mixture concentration was greater in wetland SP than in wetland E by 265 μg/L on Day 1 and by 160 μg/L on Day 28. As well, an higher initial glyphosate concentration in wetland SP (88 μg/L on Day 1) than wetland E (74 μg/L on Day 1) was observed (Degenhardt et al., 2012) while bromoxynil concentrations in both wetlands were similar on Day 1 (42 μg/L) (Degenhardt et al., 2011).

The herbicide mixture used in the present study consisted of eight herbicides, six of which had similar modes of action (MOA) to those of naturally occurring auxins (e.g., indole-3-acetic acid). Auxin-type herbicides induce synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase via increased expression of specific ACC synthase genes (Abel and Theologis, 1996; Kende and Zeevaart, 1997; Wei et al., 2000). ACC synthase is an important enzyme in the ethylene biosynthesis pathway and catalyses the conversion of *S*-adenosylmethionine to ACC

which, in turn, forms ethylene. Ethylene, a plant hormone, is responsible for plant growth regulation, epinasty, senescence, and stimulation of abscisic acid (ABA) production (Kende and Zeevaart, 1997). Although auxins, ethylene, and ABA acting together may bring about the death of plant tissue at higher concentrations, at low concentrations, growth by cell division and cell elongation is usually stimulated (Grossmann, 2000). Such stimulatory effects by toxic substances at low and sub lethal concentrations are similar to the commonly observed phenomenon, hormesis. Taken together, this evidence suggests that lower concentrations of auxin-type herbicides in wetland E likely stimulated PP and Chl *a* while relatively higher concentrations in wetland SP were likely responsible for suppression of PP and Chl *a* noted during the first week post-treatment.

There is some support for this contention in the literature. Although information on the mixture toxicity of auxin-type herbicides is scarce, what is known is that single additions of such herbicides at low concentrations tend to stimulate primary productivity. In experimental ponds, lakes and laboratory microcosms, concentrations of 2,4-D (an auxin-type herbicide) ranging from 20 to 2000 μg/L have been shown to stimulate algal primary productivity (Boyle, 1980; Kobraei and White, 1996; Wong, 2000). The reason for stimulation of PP and Chl *a* in wetland E, therefore, may be due to presence of low concentrations of the mixture of auxin-type herbicides. In the present study, the total concentration of auxin-type herbicides in both wetlands was in the range where stimulation was observed in other studies. However, stimulation was observed only in wetland E.

As noted earlier, suppression of both PP and Chl *a* was observed in wetland SP during the first week of the study. Research has shown that stimulation of algal productivity and growth by auxin-type herbicides is concentration-dependent with significant inhibition of these processes

occurring at higher concentrations (20 to 1000 mg/L – (Kobraei and White, 1996; Wong, 2000)). Although individual herbicide concentrations, detected during first week in this study, were always below those causing negative effects (Kobraei and White, 1996; Peterson et al., 1994; Wong, 2000), when added together, the concentrations may have crossed a threshold beyond which negative effects were observed as a result of concentration addition. In laboratory-based experiments, low concentrations of triazine herbicides, that individually did not cause statistically significant responses, contributed to the inhibition of algal cell reproduction when applied as a mixture (Faust et al., 2001). In the present study, the data suggest that the concept of concentration addition can be demonstrated in the field. In wetland SP, although initial suppression in PP and Chl *a* was observed, PP rates gradually increased after Day 7. This gradual increase may be indicative of the ability of pelagic phytoplankton communities to show some recovery associated with concomitant declines in herbicide concentrations with time. After Day 7, for example, herbicide concentrations (as calculated from data in Degenhardt et al. (2011)) in wetland SP decreased to levels seen in wetland E on Day 1. Even though PP and Chl *a* did increase after Day 7, rates were always lower than those on the control side perhaps indicating long-term impairment of algal productivity and growth.

Although the majority of herbicides in the mixture were auxin-type, bromoxynil and glyphosate were also present. Bromoxynil, a photosynthesis inhibitor, binds to quinine, one of the electron acceptors in photosystem II thereby preventing electron flow to plastoquinone in the electron transport system (Tomaso, 1994). Glyphosate interferes with the shikimic acid pathway disrupting synthesis of aromatic amino acids. The primary target of glyphosate, 5-enolpyruvyl shikimate 3-phosphate synthetase (EPSPase), catalyzes the formation of 5-enolpyruvyl shikimate 2-phosphate (EPSP), an intermediate in the shikimic acid pathway (Boocock and Coggins,

1983). With regards to toxic effects, laboratory studies have revealed that bromoxynil added to distilled water at 280 μg/L (about 3.5 times greater than the target concentration used in the present study) did not inhibit algal growth (Peterson et al., 1994). In an other laboratory study using algae cultured in a growth medium, glyphosate at 200 μg/L (about 2 times the target concentration used in the present study) did not affect algal growth, photosynthetic rate or Chl *a* content (Wong, 2000). Based on this evidence, the two herbicides, bromoxynil and glyphosate were likely present at concentrations too low to cause a negative effect on pelagic phytoplankton in either wetland.

In spite of high variability and lower reproducibility of field experiments, findings from the present study on wetland pelagic communities suggest that such experiments can be useful. Results also suggest the importance of considering herbicide mixture components when developing water quality guidelines. Such consideration is especially important across the prairie pothole region, where wetlands are regularly interspersed throughout the arable landscape. Mixtures of herbicides are not only used, but also detected in these ecologically important waterbodies.

5.4.2 Biofilm Communities

In both wetlands, biofilm PP and Chl *a* were initially stimulated post-treatment based on maximum-exposure scenario. Although research into effects of herbicide mixtures is limited, stimulation of PP and biomass has been observed in single herbicide exposure experiments. For example, in a river water microcosm experiment conducted in France, algal biomass and carbon incorporation rate were stimulated when biofilms were chronically exposed to 1 μg/L diuron (a photosynthesis inhibitor) (Tlili et al., 2008). In another study, Chl *a* and carbon incorporation rate increased when marine biofilms were exposed to diuron at concentrations <10 μg/L for 3 to

4 weeks (Molander and Blanck, 1992). In the present wetland study, the stimulation observed was due to the hormonal effects of the six auxin-type herbicides in the mixture as was observed for the pelagic communities. Although PP in both wetlands was initially stimulated, increases were greatest in wetland E compared to wetland SP likely due to the lower herbicide concentrations in wetland E as noted above for the pelagic community. Although stimulation did occur on day 7 in wetland SP, the higher herbicide concentrations in this wetland may have initially suppressed PP and biomass. As a result the increases over controls in wetland SP were not as great as in wetland E, where lower herbicide concentrations were observed. Although the exact mechanism of this stimulation is unknown, it might be that herbicides or their degradation metabolites within biofilms may act as inducers of quorum sensing. In this process, small molecules at concentrations less than those causing inhibition, stimulate or depress gene expression at the transcriptional level (Goh et al., 2002). According to some authors, physiological changes, like increases or decreases in productivity, reflect an initial stress response which may be an early signal of ecosystem change (Bonnineau et al., 2010).

By Day 14, a large decrease in biofilm PP and Chl *a* was observed in wetland E. One explanation might be that herbicides may have sorbed to as well as metabolized within biofilms. Atrazine and diclofop methyl, for example, along with their metabolites were detected in river biofilms grown in rotating annular bioreactors with herbicide-treated river water (Lawrence et al., 2001). Although it is unknown to what extent the metabolites of the herbicides used in this study might be toxic to biofilm microorganisms, it remains a possible explanation for at least some of the declines in algal productivity and biomass noted on Day 14. In fact, availability of contaminants in the biofilm proper has been shown to increase with decreasing biofilm density or biomass (Sabater et al., 2007). Another explanation may be associated with the large increases in

grazing invertebrates associated with the biofilms on the treatment sides of both wetlands that were noted in a concurrent study (P Messing, personal communication). In other studies, such increases have been associated with concomitant decreases in algal biomass. Invertebrate grazing on river biofilms grown in rotating annular bioreactors in the laboratory, for example, resulted in a significant reduction in both autotrophic biomass and exopolysaccharide (EPS) (Lawrence et al., 2002). Interestingly, in the present study, increases in invertebrate grazers were greatest in wetland E where the greatest decrease in Chl *a* and PP was observed from Day 7 to Day 14.

Although increased grazing can reduce autotrophic biomass and production, the process also removes overlying and senescent cells thus increasing algal cell exposure to the herbicides in the surrounding water. Muñoz et al., (2001), for example, found that invertebrate grazers accelerated suppression effects of atrazine on biofilms, grown in artificial channels using atrazine-treated well water. The processes cited above (grazing, biofilm thinning and increased exposure to herbicides within as well as outside the biofilm) may have worked in concert contributing to the large decrease in PP and Chl *a* noted on Day 14 in wetland E. By Day 21, herbicide dissipation was likely great enough that little effect on biofilm PP and Chl *a* was observed.

5.4.3 Effects on Wetland Biofilm Community Structure

Due to their sensitivity, biofilms can be used as an early warning system for detection of toxic effects on aquatic ecosystems (Bonnineau et al., 2010). In particular, pigment profiles have been used to provide 'sensitive, meaningful and quantifiable indications of ecosystem change' (Paerl et al., 2003). For example, studies in France revealed that pigment profiles of natural river biofilms were significantly different in pesticide-contaminated sites compared to pristine ones. These differences, the authors suggested, were indicative of changes in photoautotroph composition (Dorigo et al., 2007; Tlili et al., 2008). According to the pigment profiles from this study, phytoplankton community composition within the treated biofilms for both wetlands

differed from the control sides. On average, of the 12 pigments analysed for on each sampling date, differences from the control in each wetland were noted in over 80 % of pigments. Specifically, greater amounts of fucoxanthin (a pigment unique to diatoms and chrysophytes) were noted in treated biofilms in wetland E as compared to the controls. The opposite, however, was noted in wetland SP. Conversely, diatoxanthin, another pigment unique to diatoms, did not increase over control in either wetlands. This evidence suggests that the increase in fucoxanthin in wetland E may be due to chrysophytes. Chrysophytes in turn are capable of mitigating adverse effects due to their diverse nutritional strategies, ability to produce resistant resting stages as well as their versatility to switch between autotrophy, heterotrophy, and even phagotrophy (Betts-Piper et al., 2004). Based on the pigment profiles, it is evident that changes in algal community composition took place on the treated sides of both wetlands.

Within biofilms there is usually a positive relationship between algae and bacteria. Algae produce low molecular weight carbon substrates that bacteria can easily utilize for growth. As well, there is a close coupling in terms of nutrient cycling (Lawrence et al., 2004). Any stress (like exposure to herbicide mixtures) which disrupts algal community composition, therefore, has the potential to disrupt the bacterial community as well. The BIOLOG results from this study indicated that on the treated side of both wetlands there were changes in the carbon utilization patterns perhaps suggesting changes in the bacterial community in response to the herbicide mixture. Although such changes were observed, it is interesting that no significant changes in bacterial production were noted when treated biofilms were compared to the control. This lack of effect on bacterial production suggests that these wetland biofilms possess some type of functional redundancy with respect to the bacterial communities.

Pesticides have been present in prairie wetlands for many years (Cessna et al., 2006; Donald et al., 1999, 2007). As suggested by the concept of toxicant-induced succession (TIS) (Blanck, 2002; Boivin et al., 2002; Porsbring, 2009) the physio-chemical properties (including presence of toxicants) of the surrounding media select for particular organisms during biofilm development. In this fashion, chronic herbicide exposure experienced by wetland microbial communities may have gradually changed their composition resulting in a progressive replacement of sensitive species with resistant ones. BIOLOG and pigment profile data presented here, however, both indicate that exposure to the herbicide mixture, directly or indirectly, altered biofilm microbial community structure. Other research has suggested that the response of biofilm communities to pulsed acute pesticide exposures depends on whether the communities had previously been exposed to the same stressors or not (Tlili et al., 2011). In the case of prairie wetlands it may be that previous chronic herbicide exposure allows biofilm microbial communities to respond quickly and positively to intermittent exposure to high herbicide concentrations (acute exposure). The apparent recovery of the autotrophic component of the biofilms (PP and Chl *a*) by day 21 provide some support for this contention. Over long time periods, the apparent ability of wetland biofilms to respond to herbicide-induced stress may result in the development of more resistant species or those capable of herbicide degradation. Further research is required.

In summary, the present study provided evidence that the herbicide mixture at maximum-exposure scenario can exert effects on the pelagic and biofilm phytoplankton community. Hormonal (stimulatory) effects at low concentrations of auxin-type herbicides were evident. Rainfall runoff events may serve to protect phytoplankton from being affected by herbicidal effects by diluting herbicide concentrations, especially in smaller ephemeral wetlands as noted in this study. This study also demonstrated herbicidal (inhibitory) effects at low concentrations of

auxin-type herbicides as a result of concentration addition in the field. Community structural changes were also seen. Whether these changes were due to either direct or indirect effects of herbicide mixture, however, remains to be established by further investigations into inter-trophic resonance effects. Considering that most herbicides on the prairies are applied once each year, results presented here are perhaps more realistic than those derived in laboratory experiments. It is also important to note that the herbicide mixture used in this study may represent only a partial mixture of herbicides used in the prairies and also that the rates of dissipation may be considerably slower during winter months, in turn increasing persistence and thus altering the effects of herbicides on the wetland ecosystem.

6. SYNOPSIS

6.1 Background

Wetlands of prairie pothole region are important ecosystems supporting vast populations of migratory birds. These aquatic ecosystems provide habitat from both land and water making them highly productive environments. The wetlands in PPR are embedded among intensive agricultural operations where pesticides are commonly used (Donald et al., 1999; Waiser and Robarts, 1997). These pesticides eventually reach wetlands via spray drift, atmospheric deposition, surface runoff and ground water flow, putting the wetland food chain under risk of effects due to those pesticides. Among pesticides, herbicides continue to be the most frequently used ones in Canada accounting for 76 % of total pesticide sales [CropLife Canada, http://www.croplife.ca/web/english/plant_science_industry/ (accessed 3 May 2010)]. Herbicides are frequently detected in prairie water bodies (Donald and Syrgiannis, 1995; Donald et al., 1999, 2007; Waite et al., 2004) and among those detected, 2,4-D, MCPA, clopyralid, dicamba, dichlorprop, mecorpop, and bromoxynil (in the order of decreasing frequency) top the list of most frequently detected herbicides.

Even though prairie wetlands may be at risk from these herbicide mixtures, there is limited information regarding their fate and effects because most scientific research to date concerns effects of single herbicides only (Åkerblom, 2004; Brock et al., 2000). Effects on various wetland trophic levels are likely more pronounced in the presence of herbicide mixtures because of the variety of modes of action. A number of studies have found that toxic effects of mixtures usually exceed those of the individual constituents (Faust et al., 1993, 1994, 2003). As a result,

toxic effects may occur at concentrations well below the water quality guidelines because of the additive effect of individual components in the mixture (Nirmalakhandan et al., 1997).

6.2 Synthesis of Current Research

The goal of this doctoral thesis was to investigate the effects of herbicide mixtures on wetland microbial communities. To accomplish this goal a number of field-based approaches were utilized. These approaches guaranteed a degree of biological complexity (in terms of multiple trophic levels), biotic and abiotic interactions and greater ecological realism than could be found in single-species laboratory studies (Figure 6.1). Although effects on single species have been used to predict toxicity, extrapolation to how a population or community will respond is difficult. The relationship between organism survival and ecosystem integrity is extremely complex and poorly understood (Cairns and Pratt, 1989; Cairns et al., 1992) making it difficult to replicate ecosystem conditions in laboratory experiments. In spite of difficulties in replicating field studies and interpreting the resulting highly variable data, field studies that include complex environmental factors and communities, are better tools in estimating risk to various ecosystems.

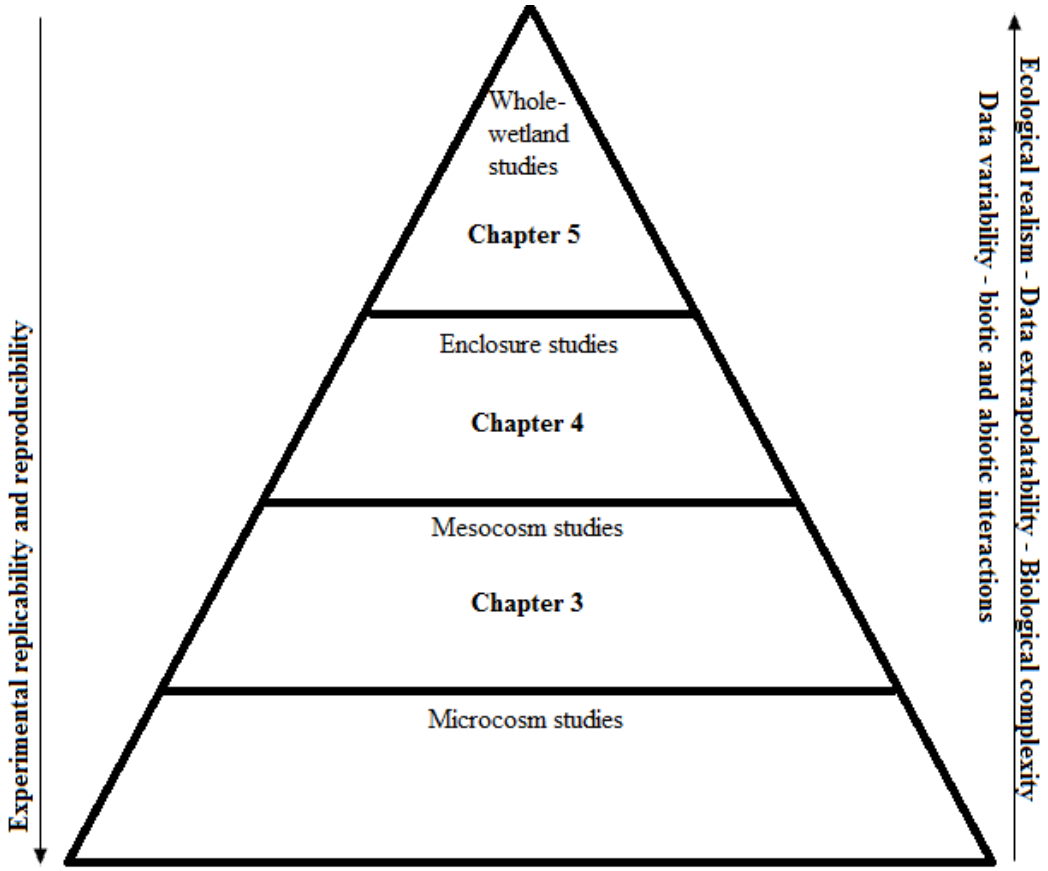


Figure 6.1 Overview of field based research with emphasis on advantages and limitations.

The strength of this thesis lies in four areas. First the herbicides investigated here are those commonly and currently found in wetlands across the prairies. Second, the herbicides used in this study are the commercially available formulations i.e., they include additives like surfactants. The majority of the toxicity studies conducted only use the technical grade herbicide to evaluate effects (Buhl et al., 1993). Thus, there is very little information on how the surfactants and adjuvants used in the commercial herbicide formulations affect the ecosystem. These additives are not generally under the same registration guidelines as pesticides (Buffington and McDonald, 2006; Hock, 1998). As well, it has been recommended that commercial formulation of herbicides be used in generating ecotoxicological data for risk assessment purposes (Waiser and Holm, 2005). Third, herbicides used in this study were investigated as

mixtures not individual herbicides. Water bodies are often contaminated with chemicals including pesticides resulting in multiple active ingredients affecting the ecosystem simultaneously. Multiple pesticides are often detected in wetlands (Donald and Syrgiannis, 1995; Donald et al., 1999, 2007) especially in those in the vicinity of agricultural farms. Fourth, this study investigates the effects using three multi-trophic field approaches including mesocosms, enclosures, and whole wetlands. These experimental units were exposed to various atmospheric processes and reflect ecological community progression and realism.

In the first study, a multi-trophic outdoor mesocosm system was used to mimic a wetland ecosystem and to investigate effects of glyphosate herbicide and two herbicide mixtures on wetland microbial communities. Mesocosms were treated with glyphosate at 1000 times the environmentally relevant concentration (ERC). One mixture consisted of six auxin-type herbicides, each at 1000 times ERC while the second mixture consisted of eight herbicides including six auxin-type herbicides, bromoxynil, and glyphosate, in dose-response additions (1, 10, 500, and 1000 times) with the ERC of each herbicide as the base concentration. Results provided evidence that the glyphosate treatment suppressed algal biomass and production for the duration of the study in both pelagic and biofilm communities. Although the auxin-type herbicide mixture initially suppressed algal biomass and production, stimulation was noted subsequently. This mixture appeared to affect microbial communities via concentration addition because of their similar modes of action of auxin-type herbicides. The eight herbicide mixture, even at low concentrations, produced negative effects on both algal and bacterial members of the wetland microbial communities.

In the second study, enclosures installed in four wetlands of differing salinities were used to investigate the effects of a mixture of eight herbicides (same as above in study one) on microbial

communities. Herbicide concentrations used here were based on a maximum-exposure scenario. Six enclosures (three controls and three treatments) were installed in each pond and primary productivity, algal biomass and bacterial production were measured in both pelagic and biofilm communities over a period of 28 days. The herbicide mixture had a stimulatory effect on primary productivity in the freshwater pond, however no such stimulatory or suppressing effects on primary productivity were observed in the saline ponds. The differences in the effects of the herbicide mixture between the freshwater and saline wetlands appear to be related to the nutrient status and salinity of the respective wetlands.

In the third study, a whole-wetland approach was used to investigate the effects of a mixture of eight herbicides (same as above in second study) at concentrations based on a maximum-exposure scenario (as in the second study). Two wetlands (one ephemeral and one semi-permanent) were selected and laminated polyvinyl curtains installed to separate each wetland into two halves (control and treated). In the two studied wetlands, the herbicide mixture had a significant effect on pelagic and biofilm phytoplankton productivity over relatively short time periods. Phytoplankton productivity in the ephemeral wetland was stimulated likely as a result of the hormonal effect of the auxin-type herbicides in the mixture. Herbicidal effects of auxin-type herbicides were noticed in the semi-permanent wetland. Here phytoplankton productivity was suppressed during the first week likely as a result of a concentration addition effect of the auxin-type herbicides in the mixture. The changes in microbial communities as a result of herbicide exposure appeared to have an effect on pond invertebrate population suggesting an inter-trophic disturbance.

It is evident from the three studies that the eight herbicide mixture had significant effects on pelagic and biofilm communities. Primary productivity was mainly affected at all concentrations

and such effects were dependent on the concentration of auxin-type herbicides. Based on the evidence in the literature (Peterson et al., 1994; Wong, 2000), two of the eight herbicides, bromoxynil and glyphosate were likely present at concentrations too low to cause a negative effect on primary productivity. Auxin-type herbicides had similar modes of action mimicking those of naturally occurring auxins. These herbicides are known to be stimulatory (hormonal) to primary productivity at low concentrations while inhibitory (herbicidal) at higher concentrations. The biphasic behaviour in the toxic effects of these herbicides was evident across all studies. Even though the concentration of individual auxin-type herbicide in the mixture was in the range where stimulation of algal growth has been reported in the literature (Boyle, 1980; Kobraei and White, 1996; Wong, 2000) inhibition in primary productivity was observed. Such inhibitory effect was likely a direct result of concentration addition of similarly acting auxin-type herbicides. The total concentration of auxin-type herbicides exceeded the concentration where hormonal effects were caused. The gradual dissipation of herbicides during the 28-day study period lowered the total auxin-type herbicide concentration in the range where they were stimulatory in effect. Because of herbicide dissipation towards the end of the study, increase in pelagic primary productivity was observed across all studies. The results in this thesis are in agreement with those found in the literature. The effects observed with auxin-type herbicide mixture were similar to those observed by individual auxin-type herbicides, for example 2,4-D, found in the literature (Boyle, 1980; Kobraei and White, 1996; Wong, 2000) providing evidence of concentration addition of auxin-type herbicides. However, such effects of auxin-type herbicides (discussed above) were dependent on the salinity of the wetlands. This may be due to the basic mechanism of binding of herbicides to ions (SO_4^{-2}, Mg^{+2}) in turn decreasing their

bioavailability to phytoplankton. In spite of scare research on this issue, there is some support for this contention in the literature (DeLorenzo et al., 2009).

Similarly, primary productivity in the biofilm communities was also stimulated at low concentrations while inhibited at higher concentrations, however, such effects were not as prominent as observed in the pelagic communities. The exopolysaccharide (EPS) produced by biofilm bacteria provided a certain degree of protection for biofilm communities (Costerton et al., 1999; Flemming, 1993). Sorption to and metabolism within biofilms of herbicides may also have effected the overall stimulatory or inhibitory effect of primary productivity in biofilms. There is evidence of herbicide sorption and metabolism within biofilms (Lawrence et al., 2001). In addition, availability of herbicides in the biofilm has been shown to decrease with increasing biofilm density or biomass (Sabater et al., 2007). Grazing invertebrates associated with the biofilms also affected the overall biofilm primary productivity. Increased invertebrate numbers as noted in a concurrent study (P Messing, personal communication), in turn may have increased grazing resulting in decreased algal biomass and productivity.

Bacterial productivity was not affected by herbicide mixture treatment suggesting that the threshold concentration of herbicides at which bacterial communities are inhibited may be higher than that for algae.

6.2.1 Herbicide Mixtures

Multiple pesticides are often detected in wetlands and among those herbicides continue to be the most frequent ones in Canada (Donald and Syrgiannis, 1995; Donald et al., 1999, 2001, 2007; Waite et al., 2004). The herbicide concentrations used to evaluate the effects of mixtures in the current thesis represent concentrations as low as those representatives of those found in prairie wetlands (environmentally relevant concentrations) as well as higher concentrations simulating direct overspray (expected environmental concentration). Wetlands interspersed in

intense agriculture farms are particularly at risk of direct overspray where some ephemeral wetlands may be cultivated in dry years.

6.2.2 Water Quality Guidelines

Pesticides including herbicides and insecticides, when present as mixtures, have been shown to cause significant negative effects even at low concentrations (Faust et al., 2001; Sura et al., 2012). These same pesticides at same concentrations did not cause significant responses when present individually (Faust et al., 2001). In the current thesis, it is demonstrated that herbicide mixture where concentrations of individual herbicides did not exceed Water Quality Guidelines for the Protection of Aquatic Life, still had significant effects on the primary productivity of wetland ecosystems. The currently established water quality guidelines developed based on single-species laboratory studies, fail to capture the synergistic or antagonistic effects of chemical mixtures as well as interaction of surrounding environment (both biotic and abiotic). Multiple pesticides are often detected in aquatic ecosystems (Donald and Syrgiannis, 1995; Donald et al., 1999, 2001, 2007; Waite et al., 2004) and concluding that concentrations of these pesticides are below water quality guidelines, undermine the overall effect of the mixtures on the ecosystem. In the light of this research as well as evidence of additive effect of pesticide mixtures (Backhaus et al., 2000, 2004; Faust et al., 2000, 2001, 2003), it is inevitable to acknowledge the presence of pesticide mixtures in aquatic environments and their additive effects and the need to reassess currently established water quality guidelines. It is important to consider the presence of other chemicals in the ecosystem with similar or dissimilar modes of action when developing water quality guidelines. In the presence of the large number of chemicals and undefined number of interactions in the environment, it may seem tedious process to develop guidelines considering mixtures; however, it may be one of the best ways to safeguard integrity and importance of ecosystems, such as sustainable wetland productivity.

6.3 Future Research

Microbial communities exposed to herbicides for longer periods can bring about permanent changes in their community structure (Dorigo et al., 2004), possibly replacing the sensitive species with resistant ones or with those capable of mineralizing the herbicide (Lancaster et al., 2009), possessing similar physiological functions in the community. Species performing similar roles (functional redundancy) in communities and ecosystems can be replaced with little disturbance on ecosystem processes (Lawton and Brown, 1993). In this thesis, the ecosystem processes such as primary and bacterial productivity assessed are insensitive to detect changes in species richness. Preserving species richness for biodiversity, functional and ecological redundancy is an important consideration for ecosystem stability and sustainability (Naeem, 1998; Walker, 1995; Walker, 1992). Herbicides in the proximity of microbial environment may exert their effect via the toxic effect of the chemical or favoring the growth of class of microbes that can degrade the chemical (Gonod et al., 2006). If there is a detrimental effect, then it may result in the decrease or complete elimination of the particular microbial class that is sensitive. But if the effect favors growth, then it may result in an increase in the microbial population that can degrade the chemical. In both instances if the effect is on only one or a limited class of microbes, then such an effect may not be measured by productivity because of the inherent capacity of the microbial community in favorable conditions to replace the lost members or make way for the thriving community. But in either case, it leads to an imbalance or shift in the communities compared to those in prestine environments. Molecular techniques such as denaturing gradient gel electrophoresis (DGGE) (Muyzer and Smalla, 1998; Tlili et al., 2008), restriction fragment length polymorphism (RFLP) (Hold et al., 2001), single-stranded conformation polymorphism (SSCP) (Oldach et al., 2000), and temperature gradient gel electrophoresis (TGGE) (Muyzer and Smalla, 1998), which generate a genetic profile or

fingerprint of microbial communities, can shed light on the changes in the species richness in communities exposed to environmental pollutants such as herbicides. These genetic material-based techniques utilize 16S and 18S ribosomal RNA (rRNA) genes of bacterial and eukaryotic organisms, respectively, to assess the species composition of a community (Dorigo et al., 2010a). Such information on microbial communities not only help in understanding effects of herbicide mixtures at structural level but also help in recovery and restoring of impacted ecosystems.

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